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Knock-out and pull-out recombineering protocols for naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*

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Summary

Phage λ Red proteins are powerful tools for pulling- and knocking-out chromosomal fragments but have been limited to the γ -proteobacteria. Procedures are described here to easily knock-out (KO) and pull-out (PO) chromosomal DNA fragments from naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*. This system takes advantage of published compliant counter-selectable and selectable markers (*sacB*, *pheS*, *gat*, and the arabinose utilization operon) and λ Red mutant proteins. *pheS-gat* (KO) or *oriT-ColE1ori-gat-ori1600-rep* (PO) PCR fragments are generated with flanking 40–45 bp homologies to targeted regions incorporated on PCR primers. One-step recombination is achieved by incubating the PCR product with cells expressing λ Red proteins and subsequent selection on glyphosate-containing medium. This procedure takes approximately 10 days and is advantageous over previously published protocols: i) smaller PCR products reduce primer numbers and amplification steps, ii) PO fragments for downstream manipulation in *E. coli*, and iii) chromosomal KO increases flexibility for downstream processing.

INTRODUCTION

The ability to manipulate the bacterial chromosome for molecular genetics, pathogenesis, and bacteria-host interaction studies is crucial for the discovery of novel vaccine, therapeutic, and diagnostic targets. The Gam, Exo, and Beta proteins of coliphage λ aid in the RecA-independent homologous recombination process to pull-out (PO) or knock-out (KO) regions from bacterial chromosomes¹. These λ Red proteins facilitate high frequency recombination between the chromosome and small homologous sequences (~40–45 bp) flanking a selectable marker. However, PO and KO manipulation of the bacterial genome

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AUTHOR CONTRIBUTION STATEMENTS

Y.K. created the constructs and performed the experiments in *B. pseudomallei*. M.H.N. performed the experiments in *B. thailandensis*. B.A.W. provided guidance for M.H.N. in this project. A.T. and P.K. isolated and sequenced the *B. pseudomallei* clinical and environmental isolates on Table 1. T.T.H. designed and supervised the experiments. Y.K., M.H.N., and T.T.H. wrote this manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

using the λ Red system has been limited to the γ -proteobacteria class^{2–5}. Therefore, protocols to expand the use of λ Red recombineering beyond the γ -proteobacteria are needed, particularly for two closely related naturally transformable β -proteobacterial species *Burkholderia thailandensis* and *Burkholderia pseudomallei*.

B. thailandensis is a relatively non-pathogenic bacterium often used as a model microbe to study various aspects of the potential bioterrorism agent *B. pseudomallei*. *B. pseudomallei* is the etiological agent of melioidosis, a globally emerging and often fatal infectious disease⁶. Work by Thongdee *et al.*⁷ has demonstrated the KO of chromosomal fragments in both naturally transformable *Burkholderia* species using PCR fragments generated by three fragment overlap-extension PCR. The requirement for large homologous regions (800–1000 bp) and overlapping PCR can be hampered by the size and G+C content of the target DNA. In addition, screening for KOs with primers annealing to chromosomal regions outside of the 800–1000 bp of homology can be difficult due to increased amplicon size. Because of these limitations and the lack of PO protocols, we developed protocols to extend the λ Red recombineering potential in these two species. Here, we present protocols for λ Red recombineering to capture or delete, for example, large chromosomal DNA fragments from two β -proteobacteria species, *B. thailandensis* and *B. pseudomallei*.

Advantages and potential beyond the presented protocols

There are general advantages to this one-step λ Red facilitated PO/KO recombineering protocol: i) arabinose selection (*araBCDEFGHI*) of pKaKa2 (Fig. 1) works in *ara-* *E. coli* and *B. pseudomallei* (Table 1); ii) the *pheS-gat FRT*-cassette, containing both selectable and counter-selectable markers, is small and can be used throughout the *Burkholderia* genus^{8,9}; iii) the use of these non-antibiotic (counter)selectable markers is in compliance with CDC/NIH guidelines and they provide efficient (counter)selection with high frequencies of positive recombinants; iv) there is no requirement to maintain and/or cure a replicating plasmid encoding Flp for *FRT*-cassette excision and the *flp*-containing PCR product incubation protocol is simple and saves time; v) it is more cost effective since multiple fragment overlap-extension PCR⁷ (can be difficult for larger DNA fragments with high G+C content) is not required in this protocol, thus reducing the number of oligos used; vi) recombination aided by λ Red proteins effectively reduces the length of homologous regions required (40–45 bp), whereas no recombination occurs without the induction of λ Red proteins (i.e. DNA incubation alone); vii) possible secondary chromosomal mutation(s) in the 800–1000 bp of homology resulting from overlap-extension PCR are avoided in λ Red recombineering which only requires 40–45 bp of homology.

There are other advantages specific to λ Red mediated *in vivo* cloning (Fig. 2). PCR amplification of the insert, which can generate mutations in the fragment to be cloned, is not required. In addition, large PCR products can be difficult to amplify, especially from the high G+C DNA of *Burkholderia* species. Using this protocol to PO large chromosomal fragments from *Burkholderia* spp. is much faster than, for example, *recA*-mediated recombination of a suicidal plasmid at the region of interest followed by restriction fragmentation, ligation, and cloning in *E. coli*¹⁰. The cloned junctions can be precise depending on primer design, and unique restriction sites can be added to the middle of the Target-PO oligos for easy downstream manipulation of *in vivo* cloned fragments. It can be envisioned that the PO protocol could generate clones of much larger or smaller fragments than the 11.6 to 31.5 kb fragments exemplified in this protocol.

Furthermore, the KO protocol could be extended to include other downstream applications. Insertional mutants may be created with the *pheS-gat FRT*-cassette such that the counter-selectable markers could be used to counterselect and generate reporter-gene fusions, in-frame non-polar deletions, and chromosomal point mutations in these β -proteobacteria, *B.*

thailandensis and *B. pseudomallei*, as previously described for other γ -proteobacteria species¹. Overall, the ability to perform these types of genetic manipulations in these species using λ Red recombineering significantly simplifies and reduces experimental labor, allowing high-throughput targeted chromosomal manipulation. For example, our lab has utilized this to knock-out over 200 targeted and confirmed mutations in one month by two graduate students.

Limitations of the present protocol

The limitations of the current protocol are that: i) there is a requirement for minimal media (typically 1× M9 glucose) supplemented with GS or cPhe during *gat* or *pheS* selection; ii) when introduced into *E. coli* at high copy number, certain PO gene products may be toxic to the cell, potentially limiting the nature of downstream applications; and iii) this protocol has been performed successfully in only the naturally transformable strains of *B. thailandensis* and *B. pseudomallei* (approximately 50% of the strains tested in this study). We have not been able to extend its use to the non-naturally transformable *Burkholderia* species (e.g. *B. pseudomallei* strain K96243 and *B. mallei* strain ATCC23344) due to potentially inefficient electrotransformation and/or restriction barriers against double-stranded DNA. Various published electroporation methods^{11–13} were tested in non-naturally transformable strains (e.g. *B. pseudomallei* K96243 and *B. mallei* ATCC23344) but no colonies or recombinants were obtained. In these non-naturally transformable species and strains, mutation could be created with other established mutagenesis procedures, as previously described^{8,9,12,14}. However, lambda-red recombineering is very broad-host-range, and we strongly believe that these tools will work in many other natural transformable strains. For example, we have extended the use of this technology successfully in nine other naturally transformable *B. pseudomallei* environmental and clinical strains (Table 1).

EXPERIMENTAL DESIGN

Genetic Constructs for Recombineering

This protocol takes advantage of our recently published *pheS* counter-selectable and *gat* selectable markers^{8,9}, along with the arabinose utilization operon (*araBCDEFGHI*)¹⁰ and the more established *sacB* counter-selectable marker¹⁴. Two new broad-host-range (bhr) replicating plasmids containing λ Red genes (*gam*, *bet*, and *exo*) that are inducible with arabinose and rhamnose, pKaKa1 and pKaKa2, respectively, were engineered for this protocol (Fig. 1a and 1b). pKaKa1 is typically used for *B. thailandensis*, using the kanamycin resistance selectable marker and the *pheS* gene as a counter-selectable marker in the presence of chlorinated phenylalanine (cPhe) as described previously⁸. Since kanamycin resistance has limited use in wildtype *B. pseudomallei* strains¹⁵, the alternative *B. thailandensis* arabinose utilization operon (*araBCDEFGHI*)¹⁰ was used as a metabolic marker for selection of pKaKa2 in *B. pseudomallei*, while the *sacB* gene was chosen for counter-selection using sucrose. The pKaKa1 and pKaKa2 plasmids contain the *Bordetella bronchiseptica* bhr origin for replication in Gram-negative bacteria¹⁶. These plasmids can be electrotransformed into *B. thailandensis* and *B. pseudomallei* or conjugated from RP4-harboring *E. coli* (e.g. E1299, Table 1), where they can eventually be cured using cPhe or sucrose after recombineering. The λ Red proteins Gam, Red β , and Red α (encoded by *gam*, *bet*, and *exo* genes) are based on optimized expression of Gam and mutated versions of Red β and Red α ¹⁷. Upon induction of the λ Red proteins, linear PCR products are naturally transformed into *B. thailandensis* or *B. pseudomallei* to generate POs or KOs. The relevant strains, plasmids, and oligonucleotides used in this protocol are shown in Tables 1 and 2.

***In vivo* PO strategy**

The *in vivo* cloning or PO protocol involves the amplification of a PCR product from the engineered plasmid pAM3G, containing a glyphosate (GS) resistance selectable marker⁹, a *bhr* origin of replication, and an origin of transfer for conjugation (Fig. 1c and 2). The PCR amplified fragment (~2.8 kb), flanked by sequences homologous to the chromosomal PO region, is incubated with cells expressing the λ Red proteins. As examples of the PO protocol, we will individually describe the *in vivo* cloning of the *B. thailandensis* arabinose utilization operon (*araABCDEFghi*, 11.6 kb)¹⁰ and the *B. pseudomallei* siderophore malleobactin biosynthetic cluster (*mba*-cluster, 31.5 kb)¹⁸ into the pAM3G replicating-plasmid backbone. The PO strategy for the *mba*-cluster is depicted in Figure 2. Positive PO frequencies of GS resistant colonies with *araABCDEFghi* clones ranged from 88% to 93% in *B. thailandensis* (Table 3), while cloning frequencies for the *mba*-cluster ranged from 90% to 100% in *B. pseudomallei* (Table 4).

Overview of KO strategy

The KO procedure utilizes a PCR amplified *pheS-gat FRT*-cassette flanked by 40–45 bp of sequence homologous to the targeted chromosomal regions (Fig. 1d). Upon introduction of the pKaKa2 plasmid and induction of the λ Red proteins' expression, KOs can be achieved in one step by transformation of the PCR-amplified *pheS-gat* cassette and selection on glyphosate. The helper plasmid pKaKa2 could be cured by counter-selection with *sacB* on sucrose-containing media (Fig. 1b). Figure 3 depicts an example of chromosomal KO using pKaKa2 in *B. pseudomallei* at high frequencies (88–92%, Table 4). We foresee that for those laboratories approved to use kanamycin-resistance selection in *B. pseudomallei*, pKaKa1, in conjunction with the *sacB-gat* cassette, could be used to generate KO in *B. pseudomallei* (Fig. 1a and 1d). In this case, pKaKa1 could be cured by counter-selection with *pheS* on cPhe-containing media.

Recycling of useful markers

There are very few selectable or counter-selectable markers for genetic manipulation in most bacteria, and the ability to reuse these precious markers in the same strain is essential. This protocol describes two strategies for recycling the *pheS-gat* or *sacB-gat FRT*-cassettes (Fig. 3). A second round of λ Red protein induction followed by incubation of cells with a short PCR fragment flanked by 40–45 bp homologous to the targeted region on the chromosome (Fig. 3) will generate an unmarked KO mutant in *B. thailandensis* or *B. pseudomallei*. This strategy can be achieved quickly because of the presence of pKaKa1 or pKaKa2 from the prior KO procedure. In an alternative strategy, which does not require λ Red proteins, the unmarked KO mutants can be generated by incubating cells with a PCR fragment containing *flp* (Fig. 3).

Overview of the Protocol

This pull-out/knock-out protocol is summarized in Figure 4. Steps 1–12 demonstrate the generation of pull-out/knock-out in *B. pseudomallei* and *B. thailandensis* (left section). Following knock-out with the *pheS/sacB-gat FRT*-cassette, unmarked mutations can be created with overlapping oligos (middle; step 13A) or FLP-mediated excision (right; step 13B). When desired, the helper plasmid can be cured via *pheS/sacB* counter-selection (step 14). The step-by-step protocol is described below.

MATERIALS

REAGENTS

Amino acids: 2,6-diaminopimelic acid (100 mg ml⁻¹ in 1 M NaOH, e.g., Acros Organics cat. no. 235540010); L-leucine (1 M filter sterilized, e.g., Acros Organics cat. no. 125121000); L-lysine (1 M filter sterilized, e.g., Acros Organics cat. no. 303340050), L-methionine (1 M filter sterilized, e.g., Acros Organics cat. no. 166160250), L-threonine (1 M filter sterilized, e.g., Acros Organics cat. no. 138930050), and L-tryptophan (1 M filter sterilized, e.g., Acros Organics cat. no. 140591000)

Bacterial Strains:

Burkholderia strains (Table 1)

Burkholderia thailandensis E264 (available from BEI Resources)

Burkholderia pseudomallei 1026b, Bp0085, Bp0091, Bp0094, Bp4001, Bp4003, Bp4122, Bp4141, Bp4144, Bp6340

! CAUTION Acquisition, possession, and manipulation of *B. pseudomallei* strains in the United States are limited to FBI screened and cleared personnel and experiments must be performed in CDC/USDA approved and registered BSL-3 select agent facilities.

E. coli strains (Table 1; available from T.T.H.)

Plasmids (Table 1; available from T.T.H.):

pKaKa1 or pKaKa2, replicating plasmids containing inducible λ Red genes (Fig. 1)

pAM3G, broad host-range plasmid encoding *gat* (glyphosate acetyl-transferase), source of PCR fragment for pull-out recombineering

pWFR-PC_{S12}-*pheS-gat* or pWFR-PC_{S12}-*sacB-gat* plasmid DNA used as template for knockout recombineering

pCD13SK-Flp-oriT-*asd*_{Ec} plasmid DNA used as template to amplify *flp* gene containing fragment

Oligonucleotides (see Table 2) (Integrated DNA Technologies)

Target-PO1 (5'-N₄₀-GATTCCTTAAGGTATACTTT-3')

Target-PO2 (5'-N₄₀-ACGGCCTCTAGGCCAGATCC-3')

Target-PO3 (N₁₆₋₂₀ identical to the 5'-end of Target-PO1)

Target-PO4 (N₁₆₋₂₀ identical to the 5'-end of Target-PO2)

Target-rev (N₁₆₋₂₀ anneals inside of the PO region, use with oligo ori1600-rep-internal to PCR confirm PO)

ori1600-rep-internal (5'-GGAAGATTTTCAGATGCTGAG-3', use with oligo Target-rev to PCR confirm PO)

Target-KO1 (5'-N₄₀-CAAGGCGATTAAGTTGGGTA-3')

Target-KO2 (5'-N₄₀-GCTCGTATGTTGTGTGGAATTGTGA-3')

Target-KO3 (N₁₆₋₂₀ identical to the 5'-end of Target-KO1)

Target-KO4 (N₁₆₋₂₀ identical to the 5'-end of Target-KO2)

Target-KO5 (5'-N₄₀-TACCCAACCTTAATCGCCTTG-3'; this oligo has identical N₄₀ as Target -KO2)

Above: N₄₀ refers to 40 bases of sequence homologous to your target gene, where underlined bases can be common to the target and also anneal to the PCR template to increase efficiency and reduce costs.

Target-up-out (N₁₆₋₂₀ anneals outside of the homologous region, use with Target-down-out to PCR confirm KO)

Target-down-out (N₁₆₋₂₀ anneals outside of the homologous region, use with Target-up-out to PCR confirm KO)

Plac-up (5'-GCCCAATACGCAAACCGCCTCTC-3')

Flp-down (5'-TAAATGGATCCTTATATGCGTCTATTTATG-3')

DMSO (e.g., Acros Organics cat. no. 295520010)

100 bp DNA ladder (range 100 to 1,500 bp, e.g., New England Biolabs, cat. no. N3231L)

1 kb DNA ladder (range 0.5 to 10 kb, e.g., New England Biolabs, cat. no. N3232L)

dNTPs (2 mM; e.g., New England Biolabs, cat. no. N0447L)

LB agar Lennox and LB broth Lennox (e.g., Teknova, cat. nos. L9330 and L9310)

LS agar (LB agar without NaCl; e.g. Teknova, cat. no. L9200)

Saccharides: L-arabinose (2 M filter sterilized; e.g., MP Biomedicals, cat. no. 10076); L-rhamnose (10% (w/v) filter sterilized; e.g., Sigma-Aldrich, cat. no. R3875); D-glucose (1 M filter sterilized; e.g., Sigma-Aldrich, cat. no. G7528); Sucrose (1 M filter sterilized; e.g., Tekanova, cat. no. S0002)

DL-chlorinated phenylalanine (cPhe; e.g., Acros Organics, cat. no. 15728-0050)

Enzyme: *Pfu* polymerase (e.g., Stratagene, cat. no. 600153)

CaCl₂ (1 M, autoclaved in water; e.g., Acros Organics, cat. no. 194635)

MgSO₄ (1 M, autoclaved in water; e.g. Acros Organics, cat. no. 194699)

M9 minimal salts (5×, e.g., Sigma-Aldrich, cat. no. M6030); alternative preparation¹⁹

Agar (molecular biology grade; e.g., Teknova cat. no. A777)

Agarose (molecular grade; e.g. Research Products International, cat. no. A20065-100.0)

Glyphosate, super-concentrated Round Up® (50% (v/v) GS from Home Depot or other farm/garden supply stores)

EQUIPMENT, SUPPLIES, AND FACILITIES

0.22 µm pore size syringe filters (25 mm diameters; e.g., Fisher Scientific cat. no. 09-719E)

1.5 ml microcentrifuge tubes (e.g., Corning cat. no. 3621)

1–200 µl beveled pipet tips (sterile; e.g., Fisher Scientific cat. no. 02-707-450)

37°C incubators

Aerosol barrier plug tips, 200 µl and 1000 µl (sterile; e.g., Genesee Scientific cat. no. 24-412 and 24-430)

Biosafety level three (BSL-3) laboratory (inspected and approved by CDC/USDA) and practices as recommended by the *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition²⁰. Although non-antibiotic markers and the kanamycin resistance marker are described in this protocol, prior approval for their use in *B. pseudomallei* must be sought with the CDC/USDA for work performed or funded by federal agencies within the United States.

Culture tubes (14 ml, sterile disposable, snap cap; e.g. BD Falcon cat. no. 352018)

DNA purification kits: plasmids isolation kit (e.g., Zyppy™ Plasmid Miniprep Kit cat. no. D4036); gel DNA recovery kit (e.g., Zymoclean™ Gel DNA Recovery Kit cat. no. D4001); DNA cleanup kit (e.g., Zymoclean™ DNA Clean & Concentrator Kit cat. no. D4003)

Electroporation cuvettes (2 mm gap; e.g., Genesee Scientific cat. no. 40-101)

Electroporator (e.g., Bio-Rad Gene Pulser Xcell with pulse control module cat. no. 165-2662)

Falcon tubes (15ml, conical, screw cap, sterile, disposable; e.g., BD Falcon cat. no. 352097)

Gradient cycler (e.g., Eppendorf Mastercycler gradient)

Inoculation loop (e.g., Fisher Scientific cat. no. 130753)

Micro-centrifuge (e.g., Eppendorf Mini-Spin Plus cat. no. 5453-000.011)

Petri dish (e.g., Fisher Scientific cat. no. 08-757-12)

Spectrophotometer (e.g., Eppendorf Biophotometer cat. no. 6131 000.020)

Vacuum concentrator (e.g., Thermal Scientific SpeedVac cat. no. DNA120-115)

REAGENT SETUP

Agarose gels

Agarose gels are prepared by adding molecular grade agarose to 100 ml of 1× TAE buffer¹⁹ to obtain desired concentrations. The mixture is then heated and allowed to cool in a gel mold. The agarose gels are prepared freshly before use.

Antibiotic stock and nutrient solution preparation

Prepare stock solutions of D-glucose (1 M), kanamycin (35 mg ml⁻¹), L-arabinose (2 M), CaCl₂ (1 M), MgSO₄ (1 M), and L-rhamnose (10%). Dissolve all compounds in the appropriate amounts of double distilled water (DDW). The arabinose, kanamycin, glucose, and rhamnose solutions should be passed through 0.2 μm syringe filters to sterilize. 5× M9 minimal salts solution is prepared by autoclaving the 5×salt mixture with the appropriate volume of DDW. The 1 M CaCl₂ and 1 M MgSO₄ can be autoclaved separately to sterilize. Store the kanamycin stock solution at 4°C. The sugar and salt solutions can be stored at room temperature for several months.

Liquid media preparation

Use LB broth to prepare rich media. To prepare 1 liter of M9 glucose (MG) medium, add 200 ml 5× M9 minimal salts solution, 20 ml 1 M glucose, 500 μl 1 M MgSO₄, 25 μl CaCl₂, and 780 ml sterile DDW. Final concentrations are: 1× M9 minimal salts, 20 mM glucose, 500 μM MgSO₄, and 25 μM CaCl₂. One liter of M9 arabinose (MA) medium is made by adding 200 ml 5× M9 minimal salts solution, 20 ml 2 M arabinose, 500 μl 1 M MgSO₄, 25 μl CaCl₂, and 780 ml sterile DDW. Final concentrations are: 1× M9 minimal salts, 40 mM

arabinose, 500 μM MgSO_4 , and 25 μM CaCl_2 . The liquid media can be made and stored at room temperature for several months.

Solid media preparation

All solid media could be made fresh or stored at 4°C for several months. Use LB or LS agar to prepare rich plate media. One liter of M9 glucose (MG) or M9 arabinose (MA) with agar is made by first preparing 15 g agar in 780 ml DDW and sterilizing. After autoclaving, add 200 ml 5× M9 minimal salts solution, 20 ml 1 M glucose or 20 ml 2 M arabinose. Let it cool to ~50 °C before adding 500 μl 1 M MgSO_4 and 25 μl CaCl_2 . The final concentrations are: 1.5% (w/v) agar, 1× M9 minimal salts, 20 mM glucose or 40 mM arabinose, 500 μM MgSO_4 , and 25 μM CaCl_2 . Approximately 50 plates could be made from 1 liter of medium. When growing *B. pseudomallei* *asd*-specific mutant in minimal medium (Table 1 and Fig. 7), Met, Lys, and Thr are added at final concentration of 1 mM, and DAP was added at 200 $\mu\text{g/ml}$. Sucrose is added at a concentration of 15% (w/v). cPhe is added at a concentration of 0.1% (w/v) as previously described⁸.

PROCEDURE

Preparing pull-out/knock-out DNA fragment • TIMING 1 d

- 1 Design oligonucleotide primers such that the last 45 bases on the 5' end are homologous to two corresponding regions on the chromosome for the pull-out/knock-out method and order oligos prior to initiating the protocol. When designing the pair of pull-out or knock-out oligos, avoid five consecutive matching base pairs between them within their 5' ends to prevent intra-fragment recombination². All oligos do not require PAGE purification, however, the majority of the long oligos (60–65 bp) produced will be truncated or have incorrect 5'-ends since the coupling or synthesis efficiency is not 100% (coupling efficiencies are available from oligo manufacturer), resulting in reduced recombineering efficiency (e.g., approximately 29% of the 60 bp oligos have truncated or incorrect ends when the coupling efficiency is 99%, or up to 95% will have truncated or incorrect ends if coupling efficiency is as low as 95%). To remedy this problem and increase recombineering efficiency at reduced cost, a two-step PCR approach is described below. The 1st PCR product is obtained using the long oligos, which is used as the template for subsequent amplification by shorter oligos (16 – 20 bp) annealing to the 5'-ends of the 1st PCR product. The shorter oligos required for the second PCR should have identical sequences as the 5'-end of the long oligos from the 1st PCR, which would repair and yield more correct 5'-ends (Fig. 1 and Table 2). ● **TIMING 1 h**
- 2 Set up one PCR reaction using the long oligos and the following components in a thin-walled PCR tube. ● **TIMING 10 min**

Component	Amount (μl)	Final
ddH ₂ O	35.0	-
10× <i>Pfu</i> buffer	5.0	1×
dNTPs (2 mM)	5.0	0.2 mM
pAM3G or pwFRT- <i>pheS/sacB-gat</i> DNA (~20 ng μl^{-1})	1.0	~20 ng
Target-PO1 or Target-KO1 (30 μM)	1.0	30 pmol

Component	Amount (μl)	Final
Target-PO2 or Target-KO2 (30 μM)	1.0	30 pmol
<i>Pfu</i> polymerase (2.5 U μl ⁻¹)	2.0	5 U

▲ CRITICAL STEP It is essential to use *Pfu* polymerase or other polymerases that generate blunt-ends to avoid the addition of unwanted bases (e.g. A at the 3'-end with *Taq*). The proofreading capability of *Pfu* polymerase will also significantly increase the fidelity of the PCR products.

3 Carry out the PCR as below: ● **TIMING 4 h**

Set the lid temperature to 98°C to prevent condensation of the sample during the reaction.

Cycle Number	Denaturation	Annealing	Extension	Termination
1	94°C, 2 min			
2–34	94°C, 30 s	58°C, 30 s	72°C, 3 min (pAM3G) or 2 min (pwFRT- <i>pheS/sacB-gat</i>)	
35			72°C, 5 min	
36				4°C, hold

■ PAUSE POINT PCR samples can be left in the PCR machine or stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

4 Visualize all PCR reactions via agarose gel electrophoresis using a 1.0% agarose gel submerged in TAE buffer (see **REAGENT SETUP**). If oligos are designed as recommended, PCR products of ~2.7 kb (*oriT-ColE1ori-gat-ori1600-rep* from pAM3G template) or ~1.8/2.3 kb (*pheS/sacB-gat* from pwFRT-*pheS/sacB-gat* template) can be expected. Purify DNA from the gel with desired kit/protocol and quantify using a spectrophotometer at 260 nm. This 1st PCR product could be used directly for recombineering in *B. thailandensis* with lower frequencies compared to the 2nd PCR product (Table 3), however, the 1st PCR product should not be used directly for *B. pseudomallei* due to the lower frequencies obtained (Table 4). ● **TIMING 2.5 h**

■ PAUSE POINT If not used for transformation immediately, the DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

5 Set up the second PCR using the product from step 4 as template. Typically, set up four PCR reactions to obtain sufficient amounts of DNA, using the shorter oligos as below. Multiply all of the following components by four and combine in a master mix. Pipet 50 μl of the master mix into four thin-walled PCR tubes. ● **TIMING 30 min**

Component	Amount (μl)	Final
ddH ₂ O	35.0	-
10× <i>Pfu</i> buffer	5.0	1×
dNTPs (2 mM)	5.0	0.2 mM

Component	Amount (μl)	Final
<i>oriT-ColEI</i> <i>ori-gat-ori1600-rep</i> or <i>pheS/sacB-gat</i> DNA (~20 ng μl ⁻¹)	1.0	~20 ng
Target-PO3 or Target-KO3 (30 μM)	1.0	30 pmol
Target-PO4 or Target-KO4 (30 μM)	1.0	30 pmol
<i>Pfu</i> polymerase (2.5 U μl ⁻¹)	2.0	5 U

▲ CRITICAL STEP It is essential to use *Pfu* polymerase or other polymerases that generate blunt-ends to avoid the addition of unwanted bases. The proofreading capability of *Pfu* polymerase will significantly increase the fidelity of the PCR products.

6 Carry out the PCR as below: ● **TIMING 4 h**

Set the lid temperature to 98°C to prevent condensation of the sample during the reaction.

Cycle Number	Denaturation	Annealing	Extension	Termination
1	94°C, 2 min			
2–34	94°C, 30 s	58°C, 30 s	72°C, 3 min (pAM3G) or 2 min (pwFRT- <i>pheS/sacB-gat</i>)	
35			72°C, 5 min	
36				4°C, hold

■ PAUSE POINT PCR samples can be left in the PCR machine or stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

7 Visualize all PCR reactions via agarose gel electrophoresis using a 1.0% agarose gel submerged in TAE buffer (see **REAGENT SETUP**). If oligonucleotides are designed as recommended, PCR products of ~2.7 kb (*oriT-ColEI**ori-gat-ori1600-rep* fragment) or ~1.8/2.3 kb (*pheS/sacB-gat* FRT-cassette) can be expected. Purify DNA from all bands produced with desired kit/protocol and quantify using a spectrophotometer at 260 nm. ● **TIMING 2.5 h ?**

TROUBLESHOOTING

■ PAUSE POINT If not used for transformation immediately, the DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

ALTERNATIVE STRATEGY If cost is not an issue, PAGE purified long oligos could be ordered and the 1st PCR product could be used directly for recombineering without the need for a 2nd PCR. The PCR with PAGE purified long oligos should be set up exactly as the 1st PCR.

Introduction of pKaKa1/pKaKa2 into *Burkholderia* species

8 The plasmids encoding λ red proteins are pKaKa1 (for *B. pseudomallei* and *B. thailandensis*) and pKaKa2 (for *B. pseudomallei*). One of these plasmids should be introduced into bacteria prior to PO/KO experiments, via electroporation or conjugation. Option A describes delivery of pKaKa1 by electroporation. pKaKa1 contains the kanamycin resistance marker and selection is performed on LB + Km500 for *B. thailandensis*, or LB + Km1000 for *B. pseudomallei*²¹.

Delivery of pKaKa2 by electroporation or conjugation is described in Option B or C, respectively. pKaKa2 confers arabinose utilization ability to *B. pseudomallei* and is selected for on MA medium. The conjugation method (Option C) is particularly useful for delivering plasmid into the select agent *B. pseudomallei*, as it reduces the risk of generating aerosols during electroporation. Although we only describe the mating protocol for pKaKa2 into *B. pseudomallei*, but conjugation of pKaKa1 into *B. pseudomallei* could be done similarly. For KO in *B. pseudomallei*, the pKaKa1 helper plasmid should be used in conjunction with the *sacB-gat* fragment, while pKaKa2 should be used with *pheS-gat* fragment.

Option A. Electroporation of pKaKa1 into *B. thailandensis* or *B. pseudomallei* • TIMING 4 d

- (i) Grow 5 ml of *B. thailandensis* or *B. pseudomallei* in LB broth from a single colony overnight at 37°C in a shaking incubator set to 225 r.p.m. ● **TIMING ~10 min; end of day 1**
- (ii) After the culture has reached a sufficient density (OD₆₀₀ 0.8–1.5), spin down all 5 ml in a mini-centrifuge at 10,000 *g* for 1 min. Wash the cell pellet with 1 ml of cold, sterile ddH₂O. Repeat this 4 more times adhering to the same centrifugation conditions. ● **TIMING ~20 min**

! CAUTION Pipette and wash gently to minimize aerosolization of *B. pseudomallei* culture. Filtered pipette tips should be used for *B. pseudomallei* to avoid contamination of the pipette.

- (iii) Remove all water from the washed pellet and resuspend the cell pellet with 40 µl of cold, sterile ddH₂O. Add 1 µl of purified pKaKa1 plasmid DNA (0.2–0.5 µg) to the competent cells and transfer the mixture to an electroporation cuvette. Place the electroporation cuvette in the electroporator shock chamber and apply a shock of 2.5 kV, 25 µF, 200 Ω. Ensure an exponential decay of the applied shock and the absence of an arc. ● **TIMING ~1 h**

? TROUBLESHOOTING

- (iv) Immediately add 1 ml of rich broth media (e.g., LB broth) to the cuvette and transfer to a round bottom culture tube. Incubate the culture for 1 h at 37°C with shaking at 225 r.p.m. to allow for kanamycin resistance gene expression (Fig. 1a). ● **TIMING ~1 h**
- (v) Pipet 100 µl of the recovery mixture onto an LB + Km500 (*B. thailandensis*) or LB + Km1000 (*B. pseudomallei*) plate while centrifuging the rest of the recovered culture at 16,000 *g* for 1 min prior to plating. Remove all but 100 µl of media, then spread the remaining culture on another LB + Km plate. Incubate both plates in a 37°C incubator until colonies appear ~2 d later. ● **TIMING ~2 d**

ALTERNATIVE STRATEGY Instead of using cold ddH₂O, room temperature 300 mM sucrose may be substituted when washing cells as previously described¹⁵. Comparable electroporation efficiencies can be obtained using either method.

Option B. Electroporation of pKaKa2 into *B. pseudomallei* • TIMING 4 d

- (i) In a 15 ml Falcon tube, grow *B. pseudomallei* in 5 ml of LB broth overnight at 37°C in a shaking incubator set to 225 r.p.m. ● **TIMING ~10 min; end of day 1**

- (ii) After the culture has reached a sufficient density (OD_{600} 0.8–1.5), spin down all 5 ml in a mini-centrifuge at 10,000 *g* for 1 min. Wash the cell pellet with 1 ml of cold, sterile ddH₂O. Repeat this 4 more times adhering to the same centrifuge conditions. ● **TIMING ~2 h**
- (iii) Remove all water from the washed pellet and resuspend the cell pellet with 40 μ l of cold, sterile ddH₂O. Add 1 μ l of purified pKaKa2 plasmid (~0.2 μ g) DNA to the competent cells and transfer the mixture to an electroporation cuvette. Place the electroporation cuvette into the electroporator shock chamber and apply a shock of 2.5 kV, 25 μ F, and 200 Ω . Ensure an exponential decay of the applied shock and the absence of an arc. ● **TIMING ~0.5 h ?**

TROUBLESHOOTING

- (iv) Immediately add 1 ml of rich broth media (e.g., LB broth) to the cuvette and transfer to a 15 ml culture tube with a screw cap. Incubate the culture for 1 h at 37°C with shaking at 225 r.p.m. for recovery. ● **TIMING ~2 h**
- (v) Transfer the recovery mixture into a 1.5 ml microcentrifuge tube and spin in a mini-centrifuge at 10,000 *g* for 1 min. Discard the supernatant, wash the cell pellet twice with 1 ml of 1 $\times$ M9 buffer, and resuspend the cell pellet in the same volume of 1 $\times$ M9 buffer. Plate 100 μ l of the resuspended cells onto an MA agar plate, while centrifuging the rest at 16,000 *g* for 1 min. Remove all but 100 μ l of buffer, then spread the remaining culture on another MA agar plate. Incubate both plates in a 37°C incubator until colonies appear ~2 d later. ● **TIMING ~2 d**

ALTERNATIVE STRATEGY Instead of using cold ddH₂O, room temperature 300 mM sucrose may be substituted when washing cells as previously described¹⁵. Comparable electroporation efficiencies can be obtained using either method.

Option C. Conjugation of pKaKa2 into *B. pseudomallei* • TIMING 3 d

- (i) Grow a single colony of *B. pseudomallei* in 2 ml of LB broth, and the *E. coli* donor harboring pKaKa2 (E1354/pKaKa2) in 2 ml of MA + 1 mM Leu + 1 mM Lys + 1 mM Met + 1 mM Thr + 1 mM Trp + 100 μ g ml⁻¹ DAP, overnight at 37°C in a shaking incubator set to 225 r.p.m. ● **TIMING ~10 min; end of day 1**
- (ii) After both cultures have reached a sufficient density (OD_{600} 0.8–1.5), spin down 1 ml of each culture separately in a mini-centrifuge at 10,000 *g* for 1 min. Remove all medium from the *E. coli* donor and all but 40 μ l LB from the *B. pseudomallei* tube, and resuspend both pellets in this 40 μ l of LB. Spot this 40 μ l mixture on the surface of a dried and prewarmed (37°C) LB agar plate, and incubate at 37°C for 4 h. ● **TIMING ~4.5 h**
- (iii) Gently scrape the cells off the plate with sterile inoculation loop and resuspend in 1 ml of 1 $\times$ M9 in a 1.5 ml microcentrifuge tube, spin in a mini-centrifuge at 10,000 *g* for 1 min. Discard the supernatant, wash the cell pellet once more with 1 ml of 1 $\times$ M9 buffer, and resuspend the cell pellet in the same volume of 1 $\times$ M9 buffer. Plate 100 μ l of the resuspended cells onto an MA agar plate, and incubate in a 37°C incubator until colonies appear ~2 d later. ● **TIMING ~2 d**

Induction of λ red protein expression • TIMING ~18 h

- 9 The λ red proteins encoded on A, pKaKa1, and B, pKaKa2 require different induction conditions as detailed below. Option A describes the induction of the λ Red proteins encoded on pKaKa1 with arabinose. Option B describes the induction of the λ Red proteins encoded on pKaKa2 with rhamnose.

Option A. Induction of the λ Red proteins encoded on pKaKa1

- (i) Grow the colonies of *B. thailandensis* or *B. pseudomallei* harboring pKaKa1 in 4 ml of LB broth containing Km 300 or 1000, respectively. ● **TIMING ~12 h**
- (ii) When the culture grows to an OD₆₀₀ of ~1.4 (*B. thailandensis*) or ~0.8 (*B. pseudomallei*), add arabinose to a final concentration of 10 mM to induce the λ red system on pKaKa1. After ~4 h of induction, harvest the culture by centrifugation. Concentrate the culture ~200 times by resuspending all cell pellets in 20 μ l of LB broth. Incubate the resuspended cells with DNA obtained in step 7 immediately. ● **TIMING ~5 h**

▲ **CRITICAL STEP** Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be “unhealthy” after induction and recombineering efficiencies decrease.

Option B. Induction of the λ Red proteins encoded on pKaKa2

- (i) Grow the colonies of *B. pseudomallei* harboring pKaKa2 in 4 ml of MA at 37°C with shaking at 225 r.p.m. ● **TIMING ~12 h**
- (ii) When the culture grows to an OD₆₀₀ of ~0.8, add rhamnose to a final concentration of 0.2% to induce the λ red system on pKaKa2. After ~4 h of induction, harvest the culture by centrifugation. Concentrate the culture ~200 times by resuspending all cell pellets in a 20 μ l volume of LB broth. Incubate the resuspended cells with DNA obtained in step 7 immediately. ● **TIMING ~5 h**

▲ **CRITICAL STEP** Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be “unhealthy” after induction and recombineering efficiencies decrease.

DNA incubation • TIMING ~4 d

- 10 Add 0.5–2.0 μ g of PO/KO DNA obtained in step 7 of the protocol, depending on the desired frequency (Tables 3 and 4), to a 20 μ l aliquot of induced and concentrated cells. Incubate the cultures for 30 min at room temperature without shaking. After 30 min add 2 ml of LB, incubate at 37°C with shaking at 225 r.p.m. for 1 h. ● **TIMING ~1.5 h**

▲ **CRITICAL STEP** A small volume of the DNA sample (10 μ l) should be added to the concentrated cells as close contact between the cells and DNA is critical for efficient DNA uptake. If the volume of the DNA sample exceeds 10 μ l, dry it down in a vacuum concentrator.

- 11 Pipet the recovery mix into a 1.5 ml microcentrifuge tube and centrifuge at 16,000 *g* for 1 min. Wash the pellet twice with 1 ml of 1 $\times$ M9. Remove 800 μ l of the 1 $\times$ M9 and resuspend the cell pellet in the remainder of the M9. Pipet and spread 50 μ l and 150 μ l of the recovery onto two different MG plates containing

0.04% GS for *B. thailandensis* or 0.3% GS for various strains of *B. pseudomallei* (with the exception of strain Bp0091 where 0.1% GS was used). Colonies should be visible in ~3 d. ● **TIMING ~3 d**

PCR Screening for successful pull-out/knock-out

- 12** The screening for correct clones for pull-out (option A) or knock-out (option B) is done differently as detailed below.

Option A. Pull-out screen in *B. thailandensis* and *B. pseudomallei* ● **TIMING ~3 d**

- (i) Single colonies, containing the pull-out fragment as a replicating plasmid, should appear with a frequency similar to those shown in Table 3 or 4 (Typically, when 0.5–2 µg PO DNA was used, 20–200 colonies should be expected for *B. thailandensis* or 20–60 colonies for *B. pseudomallei*). To verify a positive pull-out, a Target-rev gene specific oligonucleotide (e.g. araI-rev or mbaS-rev) and a pAM3G specific oligonucleotide (e.g. ori1600-rep-internal) should be used for PCR verification (Table 2 and Fig. 5). PCR verification should be set-up exactly as described above in steps 2–3 with extension time ~1 min/kb. ● **TIMING ~4 h**

? TROUBLESHOOTING

- (ii) Once a successful pull-out of the desired fragment is verified, inoculate the positive colonies into liquid MG + 0.01% GS (*B. thailandensis*) or MG + 0.1% GS (*B. pseudomallei*). Isolate the plasmid with a plasmid isolation kit and electro-transform into an *E. coli* cloning strain (e.g., EPMaX-10B or E1889 in Table 1) for maintenance and downstream manipulation. Electro-transformation of *E. coli* is described elsewhere⁸. ● **TIMING ~2 d**

? TROUBLESHOOTING

ALTERNATIVE STRATEGY Instead of purifying plasmids from liquid culture, pipet 100 µl of the culture into a 1.5 ml micro-centrifuge tube and boil the tube for 8 min. Centrifuge the tube at 16,000 *g* for 1 min to spin down the cell debris. Electroporate 5 µl of the boiled supernatant into an electrocompetent *E. coli* cloning strain (e.g., EPMaX-10B or E1889 in Table 1) for further downstream applications.

Option B. Knock-out screen in *B. pseudomallei* ● **TIMING ~5 h**

- (i) Single colonies, with the *pheS/sacB-gat* fragment recombined into the chromosome deleting the target gene, should appear with a similar frequency as shown in Tables 4 and 5 (Typically, when 0.5–2 µg KO DNA was used, 20–50 colonies should be expected). To verify a successful knock-out, a set of Target-up-out and Target-down-out oligos annealing to chromosomal regions outside of the targeted KO primers are used (e.g. mbaF-out and mbaS-out, Table 2). PCR verification should be set-up exactly as described above in steps 2–3 with extension time ~1 min/kb. ● **TIMING ~4 h**

? TROUBLESHOOTING

- (ii) Upon verification of the knock-out, it is critical to purify the positive colonies on MG + GS plate once before growth for storage. If unmarked mutations are desired, ensure the mutant still harbors pKaKa2 by growing in MA broth or pKaKa1 by growing in LB + Km1000 broth, and follow step 13 below to

remove the *pheS/sacB-gat* fragment from the mutant chromosome. ● **TIMING ~10 min**

(OPTIONAL) Recycling of the *pheS/sacB-gat* markers for KO in *B. pseudomallei* • TIMING 3 d

- 13** The *pheS/sacB-gat* fragment can be removed from the mutant obtained in step 12 by two different strategies. Option A describes replacement of the *pheS-gat* or *sacB-gat* cassette by a small PCR fragment homologous to the target gene, via λ red recombination, generating a markerless mutant. Option B describes Flp-mediated excision of the *pheS-gat* or *sacB-gat* fragment, generating an unmarked mutant with a single *FRT* scar remaining in the target gene.

Option A. Replacement of the *pheS-gat* or *sacB-gat* cassette with overlapping oligos

- (i)** Inoculate the purified colony obtained in step 12B(ii) into 4 ml of MA broth (*B. pseudomallei* with pKaKa2 and the *pheS-gat* cassette) or 4 ml of LB + Km1000 broth (*B. pseudomallei* with pKaKa1 and the *sacB-gat* cassette) and incubate at 37°C with shaking at 225 r.p.m. When the culture grows to an OD₆₀₀ of ~0.8, add rhamnose to a final concentration of 0.2% (*B. pseudomallei* with pKaKa2 and the *pheS-gat* cassette) or arabinose to a final concentration of 10 mM (*B. pseudomallei* with pKaKa1 and the *sacB-gat* cassette) to induce the λ red system. After 4 h of induction, harvest all 4 ml of culture by centrifuging at 16,000 *g* for 1 min. Discard the supernatant and resuspend the cell pellet in 20 μ l of LB. Incubate the resuspended cells with DNA obtained in step 13A(iv) below immediately. ● **TIMING ~8 h**

▲CRITICAL STEP Induction at the proper OD is crucial for efficient recombineering. If the OD is too low, toxicity of λ red proteins can cause the cells to stop growing. If the OD is too high, cells tend to be “unhealthy” after induction and recombineering efficiencies decrease.

- (ii)** Typically, set up two PCR reactions to obtain sufficient amounts of DNA, using the overlapping primers. Multiply all of the following components by two and combine in a master mix. Pipet 50 μ l of the master mix into two thin-walled PCR tubes. ● **TIMING ~30 min**

Component	Amount (μ l)	Final
ddH ₂ O	30.0	-
10× <i>Pfu</i> buffer	5.0	1×
dNTPs (2 mM)	5.0	0.2 mM
*Target-KO1 (1 μ M)	1.0	1 pmol
*Target-KO5 (1 μ M)	1.0	1 pmol
Target-KO3 (30 μ M)	3.0	30 pmol
Target-KO4 (30 μ M)	3.0	30 pmol
<i>Pfu</i> polymerase (2.5 U μ l ⁻¹)	2.0	5 U

*Target-KO1 and Target-KO5 anneal to each other and serve as template.

▲CRITICAL STEP It is essential to use *Pfu* polymerase or other polymerases that generate blunt-ends to avoid the addition of unwanted bases. The proofreading capability of *Pfu* polymerase will significantly increase the fidelity of the PCR products.

(iii) Carry out the PCR as below: ● **TIMING ~4 h**

Set the lid temperature to 98°C to prevent condensation of the sample during the reaction.

Cycle Number	Denaturation	Annealing	Extension	Termination
1	94°C, 2 min			
2–34	94°C, 30 s	58°C, 30 s	72°C, 30 s	
35			72°C, 5 min	
36				4°C, hold

■ **PAUSE POINT** At this time, the DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

(iv) Visualize PCR reactions via agarose gel electrophoresis using a 2.0% agarose gel submerged in TAE buffer (see **REAGENT SETUP**). A single PCR product of ~100 bp should be observed. Recover and purify DNA from the gel with desired kit/protocol and quantitate using a spectrophotometer. ● **TIMING ~2.5 h**

■ **PAUSE POINT** The DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

(v) Add 0.5–1.0 µg of PCR DNA (~10 µl) obtained in step 13A(iv) to the 20 µl of resuspended cells from step 13A(i). Incubate the mixture for 30 min at room temperature without shaking. After 30 min, add 2 ml of LB and incubate at 37°C with shaking at 225 r.p.m. for 2 h. ● **TIMING ~2.5 h**

▲ **CRITICAL STEP** A small volume of the DNA sample (~ 10 µl) should be added to the concentrated cells, as close contact between the cells and DNA is critical for efficient uptake of DNA. If the volume of the DNA sample exceeds 10 µl, dry it down in a vacuum concentrator.

(vi) Aliquot the recovery mix into two 1.5 ml microcentrifuge tubes and centrifuge at 16,000 *g* for 1 min. Combine the pellet and wash it twice with 1 ml of 1× M9. Remove 800 µl of the 1× M9 and resuspend the cell pellet in the remainder of the 1× M9. Spread 50 µl and 150 µl of the cell suspension onto two MG plates containing 0.1% cPhe (*B. pseudomallei* with pKaKa2 and the *pheS-gat* cassette) or two LS + 15% sucrose plates (*B. pseudomallei* with pKaKa1 and the *sacB-gat* cassette) (see **REAGENT SETUP**). Colonies should be visible in ~2 d. ● **TIMING ~2 d**(vii) To verify successful recombination and loss of the *pheS-gat* or *sacB-gat* fragment, colonies can be patched onto MG ± 0.3% GS plates to confirm GS sensitivity. The same set of oligonucleotides used in step 12B(i) (Target-up-out and Target-down-out), which anneal outside of the homologous regions, should be used for PCR verification. PCR verification should be set-up exactly as described in steps 2–3 with extension time ~1 min/kb.? **TROUBLESHOOTING**

Option B. Flp-mediated excision of the *pheS-gat* or *sacB-gat* fragment

- (i) Inoculate the purified colony obtained in step 12B(ii) into 2 ml of LB and incubate at 37°C with shaking at 225 r.p.m. Once the OD₆₀₀ reaches 0.8–1.5, harvest the cells by centrifuging at 16,000 *g* for 1 min. Discard the supernatant and resuspend the cell pellet in 20 µl of LB. Incubate the resuspended cells with DNA obtained in step 13B(iv) below immediately. ● **TIMING ~8 h**
- (ii) Typically, set up four PCR reactions to obtain sufficient amounts of DNA. Multiply all of the following components by four and combine in a master mix. Pipet 50 µl of the master mix into four thin-walled PCR tubes. ● **TIMING ~30 min**

Component	Amount (µl)	Final
ddH ₂ O	35.0	-
10× <i>Pfu</i> buffer	5.0	1×
dNTPs (2 mM)	5.0	0.2 mM
pCD13SK-Flp- <i>oriT</i> - <i>asd</i> _{Ec} (~20 ng µl ⁻¹)	1.0	~20 ng
Plac-up (30 µM)	1.0	30 pmol
Flp-down (30 µM)	1.0	30 pmol
<i>Pfu</i> polymerase (2.5 U µl ⁻¹)	2.0	5 U

▲ **CRITICAL STEP** It is essential to use *Pfu* polymerase or other polymerases that generate blunt-ends to avoid the addition of unwanted bases. The proofreading capability of *Pfu* polymerase will significantly increase the fidelity of the PCR products.

- (iii) Carry out the PCR as below:
- Set the lid temperature to 98°C to prevent condensation of the sample during the reaction.

Cycle Number	Denaturation	Annealing	Extension	Termination
1	94°C, 2 min			
2–34	94°C, 30 s	58°C, 30 s	72°C, 3 min	
35			72°C, 5 min	
36				4°C, hold

■ **PAUSE POINT** At this time, the DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

- (iv) Visualize PCR reactions via agarose gel electrophoresis using a 1.0% agarose gel submerged in TAE buffer (see **REAGENT SETUP**). A single PCR product of ~2.6 kb should be observed. Recover and purify DNA from the gel with desired kit/protocol and quantitate using a spectrophotometer. ● **TIMING ~2.5 h**

■ **PAUSE POINT** The DNA can be stored at 4°C for a short period of time. For extended storage, it is recommended that the reaction be placed in a –20°C freezer.

- (v) Add 0.5–1.0 µg of PCR DNA (~10 µl) obtained in step 13B(iv) to the 20 µl resuspended cells from step 13B(i). Incubate the mixture for 30 min at room temperature without shaking. After 30 min, add 2 ml of LB and incubate at 37°C with shaking at 225 r.p.m. for 2 h. ● **TIMING ~2.5 h**

▲ **CRITICAL STEP** A small volume of the DNA sample (10 µl) should be added to the concentrated cells, as close contact between the cells and DNA is critical for efficient uptake of DNA. If the volume of the DNA sample exceeds 10 µl, dry it down in a vacuum concentrator.

- (vi) Aliquot the recovery mix into two 1.5 ml microcentrifuge tube and centrifuge at 16,000 *g* for 1 min. Wash the pellet twice with 1 ml of 1× M9. Remove 800 µl of the 1× M9 and resuspend the cell pellet in the remainder of the 1× M9. Spread 50 µl and 150 µl of the cell suspension onto two MG plates containing 0.1% cPhe (*B. pseudomallei* with pKaKa2 and the *pheS-gat* cassette) or two LS + 15% sucrose plates (*B. pseudomallei* with pKaKa1 and the *sacB-gat* cassette). Colonies should be visible in ~2 d. ● **TIMING ~2 d**
- (vii) To verify successful *FRT* recombination and loss of the *pheS-gat* or *sacB-gat* fragment, colonies can be patched onto MG ± 0.3% GS plates to confirm GS sensitivity. The same set of oligonucleotides as in step 12B(i) (Target-up-out and Target-down-out), which anneal outside of the homologous regions, should be used for PCR verification. PCR verification should be set-up exactly as described in steps 2–3 with extension time ~1 min/kb.

? TROUBLESHOOTING

(OPTIONAL) Curing of pKaKa1/pKaKa2 • TIMING ~5 d

- 14 The pKaKa1/pKaKa2 helper plasmids can be cured from *B. thailandensis* or *B. pseudomallei* in one step. Option A describes the curing of pKaKa1 via *pheS* counter-selection and is achieved on cPhe containing media. Option B describes the curing of pKaKa2 via *sacB* counter-selection and is achieved on sucrose containing media.

Option A. Curing of pKaKa1 via *pheS* counter-selection • TIMING ~5 d

- (i) Pick 1–2 isolates of *B. thailandensis* or *B. pseudomallei* harboring pKaKa1 using a sterile inoculation loop and streak the cells out on MG + 0.1% cPhe plates. Incubate the plates at 37°C for 1–2 d until single colonies appear. ● **TIMING ~2 d**
- (ii) To confirm the loss of pKaKa1 plasmid, patch 10–20 single colonies from MG + cPhe plate onto LB + Km500 (*B. thailandensis*) or Km1000 (*B. pseudomallei*) plate and incubate at 37°C. Be sure to include *B. thailandensis* or *B. pseudomallei* harboring pKaKa1 and wildtype *B. thailandensis* or *B. pseudomallei* on the plate as positive and negative controls, respectively. ● **TIMING ~3 d**

Option B. Curing of pKaKa2 via *sacB* counter-selection • TIMING ~5 d

- (i) Pick 1–2 isolates of *B. pseudomallei* harboring pKaKa2 using a sterile inoculation loop and streak the cells out on LS + 15% sucrose plates. Incubate the plates at 37°C for 1–2 d until single colonies appear. ● **TIMING ~2 d**
- (ii) To confirm the loss of pKaKa2 plasmid, patch 10–20 single colonies from LS + sucrose plate onto MA plate and incubate at 37°C. Be sure to include *B. pseudomallei* harboring pKaKa2 and wildtype *B. pseudomallei* on the plate as positive and negative controls, respectively. ● **TIMING ~3 d**

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 6.

• TIMING

Steps 1–7, preparing KO/PO DNA: 1 d

Step 8, introduction of pKaKa1/2: 3–4 d

Step 9, induction: 18 h

Steps 10–11, DNA incubation: 4 d

Step 12, PCR screening: 1–3 d

Step 13, recycling of markers: 3 d

Step 14, curing of pKaKa1/2: 5 d

ANTICIPATED RESULTS

Pull-out recombineering efficiency of chromosomal fragments from *B. thailandensis*

Utilizing the protocol provided will typically produce 40 to 200 colonies depending on the amount of DNA and type of PCR product used (Table 3). As an example, the *B. thailandensis* *ara*-operon was pulled-out of the chromosome with a PCR fragment containing a *bhr* origin of replication. Successful removal of the *ara*-operon was verified using an *ara* specific primer and a plasmid origin specific primer (Fig. 5a). High percentages (~90%) of the colonies contained the pulled-out fragment (Table 3 and Fig. 5a). Such percentages should be common as these numbers were obtained from experiments done in triplicate and bacteria that do not contain *gat* should not grow on media containing GS.

Pull-out recombineering in *B. pseudomallei*

Using the helper plasmid pKaKa2 (constructed with the *ara*-operon pulled-out from *B. thailandensis*), the efficiencies of pull-out recombineering in *B. pseudomallei* are shown in Table 4, which are relatively lower than pull-out in *B. thailandensis* (Table 3). Introduction of pKaKa2 into *B. pseudomallei* can be achieved through electroporation or conjugation. High efficiency can be expected from both methods. Selection and maintenance of pKaKa2 in *B. pseudomallei* is simple and tight, as cells without the plasmid will not be able to metabolize arabinose as a nutrient source. A higher concentration of glyphosate (0.3% instead of 0.04% for *B. thailandensis*) should be used for selection of the pull-out plasmid. Successful pull-out of chromosomal fragments should be confirmed by PCR using two oligos, where one anneals to the plasmid backbone and the other to the pull-out fragment (e.g., *mba* cluster in Fig. 5b). High frequencies (90–100%) of successful pull-out can be expected (Fig. 5b and Table 4). No GS resistant colonies were observed if the λ Red system was not induced, indicating *recA*-mediated recombination did not recombine 40–45 bp of homology at any detectable frequencies (Table 4).

Knock-out recombineering in *B. pseudomallei* strains

We first demonstrated the KO recombineering in *B. pseudomallei* by knocking out the *mba* cluster in strain 1026b. The efficiencies of knock-out recombineering in *B. pseudomallei* *mba* cluster are shown in Table 4, where 20–50 colonies are usually obtained when 0.5–2 µg KO DNA fragment is used. Independent GS resistant isolates were screened by phenotypic and PCR confirmation (Fig. 6a and 6b), with 100% frequency of successful knock-out. Generally, high frequencies (90–100%) are expected for the successful knock-out of chromosomal fragments, when the *gat* marker is used for selection (Fig. 6a and 6b). Two options, via a second λ Red mediated recombination or Flp-catalyzed excision, were provided for the removal of the *pheS-gat* or *sacB-gat FRT*-cassette from the mutant chromosomes. The λ Red mediated recombination results in a clean deletion of the chromosome fragment, whereas the Flp-catalyzed (λ Red independent) excision leaves a single “*FRT*-scar” inside the targeted fragment. Alternatively, instead of *flp*-containing PCR product incubation, we have expressed Flp transiently by conjugating the suicidal vector, pCD13SK-Flp-*oriT*-*asd*_{Ec}, from *E. coli* Δ*asd* strain (E1354 in Table 1) and immediately counterselect the conjugation mix on cPhe- or sucrose-containing medium, which yielded GS sensitive clones. Use of the *pheS-gat* or *sacB-gat FRT*-cassette for knock-out makes the creation of unmarked mutations easy, as there is little to no resistance to cPhe or sucrose was observed. As an example, the removal of the *pheS-gat* cassette was close to 100% efficient (Fig. 6c and 6d with the *mba* cluster as an example). The confirmation of unmarked mutations is simple, as the PCR products amplified using outside primers are small (~200–500 bp; Fig. 6c and 6d). After introduction of the pKaKa2 plasmid, unmarked mutant could be obtained in approximately 10 d. pKaKa2 could be easily cured or maintained for the subsequent manipulation, such as creating multiple mutations in the same strain or introducing reporter gene fusions. If required, the curing of pKaKa2 on sucrose media could be confirmed by the inability to grow on arabinose as a sole carbon source. The curing efficiency was observed to be close to 100%. If pKaKa1 in conjunction with the *sacB-gat* fragment was used for *B. pseudomallei* KO, then the efficiencies for KO and curing of helper plasmid pKaKa1 are expected to be similar as when pKaKa2/*pheS-gat* is used. Since the first submission of this protocol, we have used this system to PO and KO genes in both species with relative ease.

To demonstrate and extend the use of λ red recombineering in other naturally transformable *B. pseudomallei* strains, we tested 20 additional *B. pseudomallei* strains and 9 were naturally transformable (Table 1). We chose these nine strains encompassing clinical and environmental isolates (Table 1). The *pheS-gat* cassette was utilized to successfully KO the *asd* gene in these nine *B. pseudomallei* strains, and results are shown in Table 5 and Figure 7. The frequencies of true mutants were high (>50%, Table 5), even for KO of the essential *asd* gene⁹, and screening two colonies should yield mutants using this system.

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Figure 1.

Genetic constructs for KO and PO recombineering in *B. thailandensis* and *B. pseudomallei*. (a) Plasmid pKaKa1 contains λ Red genes (*gam-bet-exo*)¹⁷ driven by the arabinose inducible promoter (*P_{araB}*). (b) Plasmid pKaKa2 is maintained in *ara⁻ E. coli* and *B. pseudomallei* strains by the *B. thailandensis* arabinose-utilization operon and growth on arabinose minimal medium, where λ Red genes are driven by the rhamnose inducible promoter. (c) Broad-host-range (bhr) replicating plasmid used for PO recombineering (*in vivo* cloning). Target-PO1 and Target-PO2 indicate primers (blue arrows), with 40–45 bp homology to the targeted chromosomal region, used to obtain PCR products (*oriT-ColE1ori-gat-ori1600-rep*) to PO targeted sequences. (d) The *pheS-gat* and *sacB-gat* FRT-cassettes used for KO recombineering experiments. Target-KO1 and Target-KO2 indicate primers (blue arrows), with 40–45 bp homology to chromosomal regions, used to amplify this cassette in KO experiments. **Abbreviations:** *araC_{Ec}* of pKaKa1, activator of the arabinose inducible promoter (*P_{araB}*) from *E. coli*; *araBCDEFGHI* of pKaKa2, *B. thailandensis* arabinose utilization operon¹⁰; *bla*, encodes β -lactamase; *ColE1-ori*, *ColE1* origin of replication obtained from high copy number pUC vectors²⁵; *FRT*, yeast 2 μ m plasmid Flp recombination target; *gat*, glyphosate (GS) acetyl transferase for GS resistance⁹; *Km^r*, kanamycin resistance encoded by *nptII*²⁶; *lacZ α* , encodes LacZ alpha peptide; *mob-oriT*, RP4-dependent conjugal origin of transfer of *B. bronchiseptica* cryptic plasmid pBBR1¹⁶; *ori_{1600-rep}*, *Pseudomonas* and *Burkholderia* bhr origin of replication²⁷; *ori-rep*, bhr replicon of *B. bronchiseptica* pBBR1 plasmid¹⁶; *oriT*, plasmid RP4 origin of transfer for conjugation; *P_{araB}*, arabinose inducible promoter³; *P_{lac}*, *E. coli* lactose operon promoter; *P_{rhaBAD}*, rhamnose inducible promoter²⁸; *P_{S12}* and *PC_{S12}*, constitutive promoters of *B. pseudomallei*

and *B. cenocepacia rpsL* gene²⁹; *pheS*, engineered gene encoding a mutant version of α -subunit of phenylalanyl tRNA synthase⁸; *rhaR* and *rhaS*, regulators of the rhamnose inducible promoter²⁸; *sacB*, encoding for a modified levansucrase counter-selectable marker¹⁴.

Figure 2.

Overview of an example of PO recombineering in *B. pseudomallei*. The 31.5 kb *mba*-cluster was pulled-out of the *B. pseudomallei* chromosome, using a PCR amplified fragment of the pAM3G backbone (Fig. 1c), with correctly oriented 40–45 bp homologous sequences toward the *mbaF* and *mbaS* genes. Positive clones can be immediately obtained in one step via glyphosate resistance selection. The pAM3G-*mba* bhr replicating plasmid could be screened (with the primers indicated by arrows) and isolated from glyphosate resistant *B. pseudomallei* colonies and transformed into *E. coli* for further characterization. Red crosses indicate λ-red mediated homologous recombination.

Figure 3.

KO recombineering strategy. The example shown here involves the amplification of the *pheS-gat* cassette with Target-KO primers (Fig. 1d) to generate a 1.8 kb PCR product containing 40–45 bp homologous sequences to the end of *mbaF* and the beginning of *mbaS* genes. Upon uptake by the natural transformation system of *B. pseudomallei* and selection with GS, λ Red proteins facilitate the one-step recombination of the PCR product to KO the 31.4 kb *mba*-cluster on chromosome I. The resulting *B. pseudomallei* marked mutant strain ($\Delta mba::pheS-gat-FRT$), still containing pKaKa2, is re-induced to express λ Red proteins a second time to create unmarked mutants. By transforming a smaller PCR fragment containing 40–45 bp homologies to *mbaF* and *mbaS* for recombination, unmarked mutants lacking *pheS-gat* can be easily selected by counter-selection on cPhe-containing medium. Alternatively, the *pheS-gat-FRT* cassette can be easily removed by naturally transforming a *flp*-containing PCR fragment for transient expression. The black horizontal arrows indicate outside primers used to screen for mutants.

Figure 4.

λ red recombineering scheme for PO/KO in *B. thailandensis* and *B. pseudomallei*. Strategy for PO/KO recombineering is shown on the left, whereas the middle right sections present the strategies for creating unmarked mutants via λ red recombination or FLP-mediated excision, respectively. Detailed descriptions for each step are provided in the procedure. In red are numbers that correspond to the steps in the protocol.

Figure 6.

Phenotypic and PCR confirmation for the *mba* cluster knock-outs in the *B. pseudomallei*. As an example, the *B. pseudomallei* strain 1026b *mba* cluster was knocked-out, using the *pheS-gat* fragment with 45 bp homologous sequences to the *mbaF* and *mbaS* genes. Twenty five independent knock-out mutants were spotted onto CAS plate³⁰, along with the wildtype 1026b strain (+) and the enterobactin negative *E. coli* mutant (–, JW0586-1³¹) as the positive and negative control, respectively. Since the *mba* cluster is involved in malleobactin synthesis and secretion, knock-out mutants display the malleobactin-negative phenotype as an absence of orange-halo. The knockout frequency is 100% in these twenty five isolates. (b) Oligos annealing outside of the homologous region were used to screen ten isolates from (a) for successful KOs (oligos *mbaF*-out and *mbaS*-out as in Fig. 3). 100% (10 out of 10) of the GS resistant colonies were shown to have the *pheS-gat* fragment inserted into the *mba*-cluster, as indicated by the arrow. NC indicates negative control using wildtype 1026b as the PCR template. L indicates 1 kb DNA ladder from New England Biolabs. Following the second λ Red mediated recombination with overlapping oligos (c) or FLP-mediated excision (d), unmarked mutations were generated. PCR confirmation was done for both methods, using the same outside oligos as in (b), yielding smaller PCR products lacking *pheS-gat* as indicated by arrows in (c) and (d). In both (c) and (d), L indicates 100 bp DNA ladder from New England Biolabs. (e) After mutant construction, the pKaKa2 helper plasmid was cured from the 1026b- $\Delta mba::pheS-gat$ via *sacB* counter-selection on sucrose-containing media. Total nucleic acids were purified from strain 1026b- $\Delta mba::pheS-gat$ /pKaKa2 (lane 1) and four isolates of cured strain 1026b- $\Delta mba::pheS-gat$ without pKaKa2 (lanes 2–5) for Southern hybridization analysis using the entire pKaKa2 plasmid as a probe. As indicated by arrow, the helper plasmid pKaKa2 only exists in strain 1026b- $\Delta mba::pheS-gat$ /pKaKa2,

while the entire pKaKa2 sequence is completely lost in 1026b- $\Delta mba::pheS-gat$ after curing. L, pre-biotinylated DNA ladder from New England Biolabs (mixture of HindIII digested λ DNA and HaeIII digested ϕ 174 DNA). (f) To demonstrate the integrity of the PO fragment, boiling preps of two 1026b/pAM3G-*mba* isolates were electroporated directly into 1026b-*mba::FRT* mutant for the complementation study (B1 and B2). In addition, the same boiling preps of 1026b/pAM3G-*mba* were transformed into an *E. coli* restriction-minus strain, plasmids were then re-isolated from *E. coli* and reintroduced to complement the 1026b-*mba::FRT* mutant (E1 and E2). These four complemented strains were spotted on a CAS plate along with positive (+, wildtype 1026b) and negative (–, 1026b-*mba::FRT*) controls, and all four isolates regained the ability to produce malleobactin. (g) Boiling preps from five different isolates of 1026b/pAM3G-*mba* were electroporated into *E. coli* JW0586-1 strain, and the resulted strains were spotted onto CAS plates with positive (+, *E. coli* K-12 strain) and negative (–, *E. coli* JW0586-1 strain) controls. As shown, the *B. pseudomallei* 1026b *mba*-operon complemented the enterobactin-deficient phenotype of *E. coli* JW0586-1 strain.

Figure 7.

KO recombineering in different *B. pseudomallei* strains. (a) Various *B. pseudomallei* clinical and environmental isolates were incubated with *gfp*-containing PCR product to confirm their natural competency. After DNA incubation and 45 min recovery in LB, transient expression of GFP was observed for all nine naturally transformable strains (Table 1) and representative images are shown for one of these strains. The other 11 *B. pseudomallei* strains tested did not show fluorescent bacteria and presumably are not naturally competent (data not shown). Abbreviation: DIC, differential interference contrasts. White scale bars equal 10 μ m in length. (b) Successful KO of the *asd* gene in 1026b strain and the nine newly identified naturally transformable *B. pseudomallei* strains were confirmed by PCR with oligos annealing outside of the *asd* gene on the chromosome, and one isolate from each strain is shown. W: wildtype; M: mutant; L, 1 kb DNA ladder from New England Biolabs. The ~2.1 kb PCR products obtained in all mutant strains are results of *pheS-gat* insertion in the *asd* region indicated by the ~1.1 kb PCR fragment in the corresponding wild-type strains.

TABLE 1

Strains and plasmids utilized in this study.

Strains/plasmids	Lab ID ^a	Accession Number ^b	Relevant Features and Use	Reference
<i>E. coli</i>				
EPMax10B	E1231	-	F- λ -mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR recA1 endA1 araD139 Δ (ara, leu)7697 galU galK rpsL nupG	BioRad
EPMax10B-lacI ^f /pir/leu ⁺	E1889	-	F- λ -mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR recA1 galU galK rpsL nupG lacI _q -FRT8 pir-FRT4	^c
EPMax10B-pir116/ Δ asd ^f mob-Km ^f / Δ trp::Gm ^f	E1354	-	Gm ^f , Km ^f ; F- λ -mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80dlacZ Δ M15 Δ lacX74 deoR recA1 endA1 araD139 Δ (ara, leu)7697 galU galK rpsL nupG Th-pir116-FRT2 Δ asd::wFRT Δ trp::Gm ^f FRT5 mob[recA::RP4-2 Tc::Mu-Km ^f]	^c
MC4100- Δ asd::FRT ^f mob::mFRT	E1299	-	F ⁻ (argF ⁻ lac) U169 araD139 rpsL150 relA1 thiA ptsF25 deoC1 flbB5301 rbsR Δ asd::FRT recA:RP4-2Tc::Mu	
<i>B. pseudomallei</i> ^d				
1026b	B004	-	Wildtype strain; clinical melioidosis isolate	22
1026b- Δ mba::pheS-gat	B023	-	GS ^f ; 1026b with pheS-gat-FRT fragment replacing the mba cluster	This study
1026b- Δ mba::FRT	B025	-	1026b with FRT-sequence inserted into the mba cluster	This study
1026b- Δ mba	B027	-	1026b with mba cluster deleted	This study
Bp0085	B040	-	Wildtype strain; clinical strain from a sepsis case (fatal) in Thailand, 2006	This study
Bp0085- Δ asd::pheS-gat	B079	-	GS ^f ; Bp0085 with pheS-gat-FRT fragment replacing the asd gene	This study
Bp0091	B042	-	Wildtype strain; clinical strain from a sepsis case (fatal) in Thailand, 2006	This study
Bp0091- Δ asd::pheS-gat	B080	-	GS ^f ; Bp0091 with pheS-gat-FRT fragment replacing the asd gene	This study
Bp0094	B044	-	Wildtype strain; clinical strain from a sepsis case (fatal) in Thailand, 2006	This study
Bp0094- Δ asd::pheS-gat	B081	-	GS ^f ; Bp0094 with pheS-gat-FRT fragment replacing the asd gene	This study
Bp4001 ^e	B054	-	Wildtype strain; environmental isolate from soil in Australia, 1997	This study
Bp4001- Δ asd::pheS-gat	B082	-	GS ^f ; Bp4001 with pheS-gat-FRT fragment replacing the asd gene	This study
Bp4003 ^e	B058	-	Wildtype strain; clinical strain from a sepsis case (fatal) in Australia, 1999	This study
Bp4003- Δ asd::pheS-gat	B083	-	GS ^f ; Bp4003 with pheS-gat-FRT fragment replacing the asd gene	This study
Bp4122	B064	-	Wildtype strain; environmental isolate from soil in Australia, 2006	This study

Strains/plasmids	Lab ID ^a	Accession Number ^b	Relevant Features and Use	Reference
Bp4122- Δ <i>asd::pheS-gat</i>	B084	-	GS ^r ; Bp4122 with <i>pheS-gat-FRT</i> fragment replacing the <i>asd</i> gene	This study
Bp4141 ^e	B066	-	Wildtype strain; clinical isolate from a chronic case (survived) in Australia, 1991	This study
Bp4141- Δ <i>asd::pheS-gat</i>	B085	-	GS ^r ; Bp4141 with <i>pheS-gat-FRT</i> fragment replacing the <i>asd</i> gene	This study
Bp4144	B068	-	Wildtype strain; clinical isolate from a chronic case (survived) in Australia, 1995	This study
Bp4144- Δ <i>asd::pheS-gat</i>	B086	-	GS ^r ; Bp4144 with <i>pheS-gat-FRT</i> fragment replacing the <i>asd</i> gene	This study
Bp6340 ^e	B078	-	Wildtype strain; clinical isolate from a chronic case (survived) in Australia, 2003	This study
Bp6340- Δ <i>asd::pheS-gat</i>	B087	-	GS ^r ; Bp6340 with <i>pheS-gat-FRT</i> fragment replacing the <i>asd</i> gene	This study
<i>B. thailandensis</i>				
E264	E1298	-	Prototroph; environmental isolate	23
Plasmids				
pAM3G	E1947	GU074522	GS ^r ; broad-host-range cloning vector based on GS resistance	This study
pwFRT- <i>pheS-gat</i>	E2336	GU074523	GS ^r , Ap ^r ; <i>pheS-gat</i> fragment flanked by wildtype <i>FRT</i> s	This study
pwFRT- <i>gat-sacB</i>	E2456	GU450326	GS ^r , Ap ^r ; <i>sacB-gat</i> fragment flanked by wildtype <i>FRT</i> s	This study
pKaKa1	E2273	GU074524	Km ^r ; broad-host-range λ -red helper plasmid based on Km resistance	This study
pKaKa2	E2334	GU074525	broad-host-range helper plasmid for λ -red recombineering	This study
pCD13SK-Flp- <i>oriT-asd_{Ec}</i>	E1827	EU626138	Suicidal plasmid containing Flp	24

^aPlease use Lab ID when requesting *E. coli* strains and plasmids.

^bGenbank accession number.

^cDetails on the construction of these strains/plasmids will be published elsewhere.

^dAcquisition, possession, and manipulation of these strains in the United States are limited to FBI screened and cleared personnel and experiments must be performed in a CDC/USDA approved and registered BSL-3 select agent laboratory.

^eMultiple-contig shotgun assemblies from these strains are available upon request.

TABLE 2

Oligonucleotides utilized in this study.

Number/Name	Sequence ^a
#1178; araA-PO1	5'- <u>CGCGCGGGAGATCTACGGGACGGCCCAATAGCTGCGCTCAGATT</u> CCTTAAGGTATACTTT-3'
#1179; araI-PO2	5'-GAAGCGGGCTGCGCGAA <u>CGCCGGCGTGAAGCGCGGGCCGGACGGCCT</u> CTAGGCCAGATCC-3'
#1227; araA-PO3	5'-CGCGCGGGAGATCTAC-3'
#1228; araI-PO4	5'-GAAGCGGGCTGCGCGAA-3'
#271; ori1600-rep-internal	5'-GGAAGATTTCAGATGCTGAG-3'
#1161; araI-rev	5'-TTCGTGTTTCGGGATGAAG-3'
#1187; mbaF-PO1	5'-AAGAACGCGAGCTCGGCGCCGAGCGCTTCGAGTTGATCGAGATT <u>CCTTAAGGTATACTTT</u> -3'
#1188; mbaS-PO2	5'-GCGGCACTTCGCTCGTCCATCGTTGCCGGCCGCGCAACGAACGGCCTCTAGGCCAGATCC-3'
#1125; mbaF-PO3	5'-AAGAACGCGAGCTCGG-3'
#1126; mbaS-PO4	5'-GCGGCACTTCGCTCGT-3'
#1219; mbaF-KO1	5'-GCAGCGCCGCTTGCCGCGCGCTTTGGGCTCGACGGGGCGGCAAGGCGATTAAAGTTGGGTA-3'
#1220; mbaS-KO2	5'-GCCTGCGGCGCGCACGCCGGGCGCGCGCGAGCACGGCGCGCTCGTATGTTGTGTGGAATTGTGA-3'
#1223; mbaF-KO3	5'-GCAGCGCCGCTTGCCG-3'
#1224; mbaS-KO4	5'-GCCTGCGGCGCGCAC-3'
#1229; mbaS-KO5	5'-GCCTGCGGCGCGCACGCCGGGCGCGCGCGAGCACGGCGCGCTCGTTACCCAACTTAATCGCCTTG-3'
#560; Plac-up	5'-GCCCAATACGCAAACCGCCTCTC-3'
#566; Flp-down	5'-TAAATGGATCCTTATATGCGTCTATTTATG-3'
#1221; mbaF-out	5'-GCCGCGCGTTACCGAAG-3'
#1222; mbaS-out	5'-CTTCGAACGGGGCGTTTG-3'
#1189; mbaS-rev	5'-GACGAGCTTCACGAACAC-3'
#1484; asd-KO1	5'-TTCAGCACCAGCAACGCGGGCGGCAAGGCGCCGTCGTTTCGCGGATAACAATTTACACAG-3'
#1485; asd-KO2	5'-CGCCACCCATCGCGAGCTTGCGCAGGCGGCCGACCGGCACCGACGTTGTAAAACGACGGC-3'
#1486; asd-KO3	5'-TTCAGCACCAGCAACGCG-3'
#1487; asd-KO4	5'-CGCCACCCATCGCGAGCT-3'
#1488; asd-up	5'-ATCTGATCGAGCCGGTG-3'
#1489; asd-down	5'-GTAAATGCCGACAGGTAT-3'

^aUnderlined are homologous sequences to targets on the chromosome. Shaded sequences in the long oligos are identical to the shorter oligos used in the second PCR (step 5 of the protocol). In blue are reverse complementary sequences of mbaF-KO1 and mbaS-KO5 where these primers are used to generate ~100 bp PCR product to create the unmarked mutation (step 13A).

TABLE 3

Pull-out recombineering efficiencies of the *ara*-operon in *B. thailandensis*^a.

DNA amount	1 st PCR Product ^b	2 nd PCR Product ^c
2 µg	41 ± 9 (92%)	245 ± 40 (89%)
0.5 µg	3 ± 1 (92%)	19 ± 3 (90%)
0.1 µg	1 ± 1(100%)	4 ± 0.3 (93%)

^aEach experiment was performed in triplicate, and average number of GS resistant colonies is shown with standard error of the mean. On average, 12 GS resistant colonies were PCR screened for positive pull-out and the percentage is shown in parenthesis.

^bThe 1st PCR product, amplified with the non-PAGE purified long oligos, was directly used for PO.

^cThe 2nd PCR product, amplified using shorter non-PAGE purified oligos and the 1st PCR product as template, was used for PO.

TABLE 4

Pull-out /knock-out recombineering efficiencies of the *mba* cluster in *B. pseudomalleri*^a.

DNA amount	Pull-out		Knock-out	
	Uninduced	Induced	Uninduced	Induced
2 µg	0	55 ± 11 (90%)	0	45 ± 8 (92%)
0.5 µg	ND	19 ± 5 (100%)	ND	21 ± 7 (88%)
0.1 µg	ND	3 ± 1 (100%)	ND	5 ± 2 (92%)

^a Each experiment is performed in triplicate, and average number of GS resistant colonies is shown with standard error of the mean. On average, 12 GS resistant colonies were PCR screened for positive PO/KO and the percentage is shown in parenthesis.

ND, not determined.

Knock-out recombineering efficiencies of the essential *asd* gene in various naturally transformable clinical and environmental *B. pseudomallei* isolates^a.

TABLE 5

λ -red	1026b	Bp0085	Bp0091	Bp0094	Bp4001	Bp4003	Bp4122	Bp4141	Bp4144	Bp6340
Uninduced	0	0	0	0	0	0	0	0	0	0
Induced	144 (100%)	22 (76%)	34 (88%)	15 (80%)	13 (69%)	26 (85%)	89 (53%)	52 (82%)	64 (62%)	23 (87%)

^aTwo μ g of DNA (*pheS-gat* cassette flanked by *asd*/homologous regions) was used for each incubation. Mutants were selected on MG + GS plates supplemented with 1 mM of Met, Thr, and Lys, and 200 μ g/ml DAP specific for this mutation. GS was used for *gat*-selection at final concentration of 0.3% (w/v) in all strains, with the exception of 0.1% GS used for Bp0091. Twenty GS resistant colonies were tested phenotypically and by PCR confirmation with external chromosomal primers, and the frequencies of true mutants are shown in parentheses.

TABLE 6

Troubleshooting table.

STEPS	PROBLEM	POSSIBLE REASONS	POSSIBLE SOLUTIONS
7	No second PCR product or high level of nonspecific products is obtained.	1 High-GC fragment causes inefficient denaturation and non-specific annealing.	1 Try a gradient PCR with annealing temperature range from 50–70°C, include 5–10% DMSO in the PCR reaction to aid amplification of high-GC fragments.
		2 Majority of the long primers is truncated (not PAGE purified) in this batch, resulting in first PCR products with truncated ends.	2 Inform primer synthesis company and resynthesize the non-PAGE purified long primers free of charge (from IDT etc.), and retry the PCR.
		3 Poor design of primers.	3 Redesign the primers so that their GC% is 50–60% with no significant primer-dimer and hairpin structure formation, and the amplicons are as small as possible.
8A(iii) 8B(iii)	Arcing is observed when applying electropulse.	Inefficient washing results in high salt concentration, or too much DNA is used.	Perform additional washes to the cell pellet, and/or use reduced amount of DNA (e.g. 0.5–1 µg instead of 2 µg).
12(i) 13(vii)	No PCR product or high level of nonspecific products is obtained.	1 High-GC fragment causes inefficient denaturation and non-specific annealing.	1 Try a gradient PCR with annealing temperature range from 50–70°C, include 5–10% DMSO in the PCR reaction to aid amplification of high-GC fragments.
		2 Poor design of primers.	2 Redesign the primers so that their GC% is 50–60% with no significant primer-dimer and hairpin structure formation, and the amplicons are as small as possible.
		3 The clones do not contain the desired KO or PO fragments.	3 Retry the recombineering.
12A(ii)	No colonies are obtained	Size of the PO fragments and toxicity of their gene products may be an issue.	Use a fragment containing a low copy number origin for PO (e.g., pBBR-based origin).