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Targeted Mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through Natural Transformation of PCR Fragments▽†

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***Burkholderia pseudomallei* is the causative agent of melioidosis, an overwhelming, rapidly fatal septic infection, and *B. thailandensis* is a closely related, less virulent species. Both organisms are naturally competent for DNA transformation, and this report describes a procedure exploiting this property for the rapid generation of marked deletion mutations by using PCR products. The method was employed to create 61 mutant strains. Several selectable elements were employed, including elements carrying *loxP* and *FRT* recombinase recognition sites to facilitate resistance marker excision. Chromosomal mutations could also be transferred readily between strains by transformation. The availability of simple procedures for creating defined chromosomal mutations and moving them between strains should facilitate genetic analysis of virulence and other traits of these two *Burkholderia* species.**

Burkholderia pseudomallei and *Burkholderia thailandensis* are closely related gram-negative bacteria widely distributed in soils of Southeast Asia and northern Australia (19, 21). *B. pseudomallei* is the causative agent of melioidosis, whereas *B. thailandensis* is rarely associated with human disease but can kill rodents at high infectious doses (14, 22). The *B. thailandensis* genome is smaller than that of *B. pseudomallei* (6.7 versus 7.2 Mbp) but encodes homologues of many of the established *B. pseudomallei* virulence determinants, including type III secretion systems and functions responsible for cell-to-cell spread during infection (15, 16, 20). Phylogenomic comparisons imply that the two species share numerous additional traits as well (10, 23). Thus, *B. thailandensis* serves as a low-virulence surrogate for studying numerous physiological and pathogenic characteristics of *B. pseudomallei*.

Progress in the genetic analysis of *B. thailandensis* and *B. pseudomallei* has been limited by the lack of a general procedure for creating targeted mutations based on the genome sequences. Standard two-step plasmid-based procedures (18) employing *sacB* as a counterselective marker have not been generally successful for the two species due to the presence of endogenous *sacB* genes (although there have been exceptions) (6, 12). A simpler alternative, the direct generation of predefined mutations by transformation of PCR fragments, has greatly facilitated the genetic analysis of several bacterial species (7, 11, 17). In general, the fragments carry selectable markers flanked by regions of homology oriented such that homologous recombination replaces genomic sequences with the selectable marker. In this study, we developed such a procedure for *B. thailandensis* and *B. pseudomallei* which exploits the discovery, presented here, that these bacteria can be rendered naturally competent for DNA transformation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thailandensis* E264 (from Don Woods, University of Calgary) and S95019 (Siriraj Hospital collection) and *B. pseudomallei* 1026a and 1026b (from Sharon Peacock, Wellcome Trust Unit, Bangkok, Thailand) were the parent strains used in this study. Two E264 mutants (in *gltB* and BTH_I1592) were generated by IS*lacZ*/hah insertion (9). Other mutant strains constructed in the course of these studies are listed in Table S1 in the supplemental material. Cre-*loxP* and FLP-*FRT* recombination to remove antibiotic resistance determinants was carried out by transiently introducing the appropriate recombinase genes (either by conjugal introduction of a nonreplicating plasmid or growth followed by curing of a replication-conditional [temperature-sensitive] plasmid) (2, 5). Bacteria were maintained on LB agar containing 0.8% NaCl. For transformation, bacteria were grown in a defined medium (DM) consisting of 0.25× M63 (13) supplemented with 0.2% glucose, 0.4% glycerol, 1 mM $MgSO_4$, thiamine (1 μg/ml), and six amino acids (leucine, isoleucine, valine, tryptophan, glutamic acid, and glutamine) (40 μg/ml [each]). Growth of transformants was selected on LB agar supplemented with tetracycline (50 μg/ml) or trimethoprim (100 μg/ml). All work with *B. pseudomallei* was carried out at Siriraj Hospital (Bangkok, Thailand).

DNA. DNA primers were purchased from IDT (Coralville, IA), Sigma-Aldrich Corp. (St. Louis, MO), or Operon Biotechnologies GmbH (Cologne, Germany) and are listed in Tables S2 and S3 in the supplemental material. Chromosomal DNA was purified using a DNeasy blood and tissue kit (Qiagen) and served as a template for amplifying the regions flanking genes targeted for deletion and for direct transfer of chromosomal markers between strains. Plasmid pIT2 (9) and pUC18-mini-Tn7T-Tp (4) DNAs were isolated using a QIAprep spin miniprep kit (Qiagen) and served as templates for amplifying the tetracycline and trimethoprim resistance genes.

Generation of mutagenic PCR products. Mutagenic PCR fragments were created by joining three fragments corresponding to the regions flanking the sequence to be deleted and a gene encoding antibiotic resistance (Fig. 1). The flanking sequences were derived from the published *B. thailandensis* (E264) and *B. pseudomallei* (K96243) genome sequences. The flanking regions were amplified from genomic DNA by use of 15- to 21-nucleotide primers (pairs 2F/2R and

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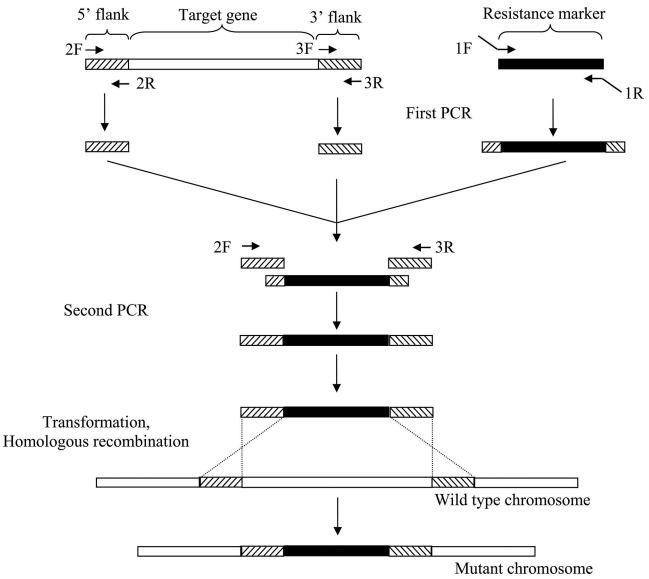

FIG. 1. Generation of defined deletions by use of PCR fragment transformation. PCR fragments designed to replace chromosomal genes with antibiotic resistance elements were generated using two sets of amplifications. The first set of amplifications generated fragments (generally about 800 bp) corresponding to the regions adjacent to the gene to be deleted (using primer pairs 2F/2R and 3F/3R) and an antibiotic resistance cassette with 50-bp end sequences identical to the adjacent region fragments (using primer pair 1F/1R). In a second amplification, the three PCR products were joined and amplified (using primer pair 2F/3R). Fragments were mixed with competent *B. thailandensis* or *B. pseudomallei* cells to allow uptake and incorporation into the genome by homologous recombination, and recombinants were selected using antibiotic resistance.

3F/3R) designed in most cases to generate approximately 800-bp fragments. DNA elements carrying antibiotic resistance markers (*tet* or *tmp*) were amplified from plasmid DNA templates pIT2 (9) and pUC18-mini-Tn7T-Tp (4), using 70-nucleotide primers comprised of 20-base 3′ segments designed to amplify the resistance elements and 50-base 5′ segments corresponding to the ends of the flanking sequences for the sites targeted (Fig. 1). Amplifications were carried out in 20-μl volumes containing 6.25 pmol of each forward and reverse primer, 0.1 μl FailSafe PCR enzyme mix E (Epicenter Technologies, Madison, WI), 10 μl FailSafe PCR 2× premix, and 1 ng plasmid DNA (for the resistance gene) or 1 ng chromosomal DNA (for sequences flanking the targeted region). The amplification conditions used for all were as follows: 95°C for 2 min (hot start); 30 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 2 min; and a final 72°C extension for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and purified using QIAquick PCR purification kits (Qiagen). The three fragments were joined through an amplification reaction which included each fragment (approximately 1 ng of each) and primers 2F and 3R (6.25 pmol of each). The cycling conditions were as described above, except that the extension time was 4 min instead of 2 min. PCR products were again analyzed using agarose gel electrophoresis, and bands of appropriate sizes were excised and extracted with a QIAquick gel extraction kit (Qiagen). These fragments (ca. 1 ng) were used as templates for additional amplification using primers 2F and 3R under the conditions used in the previous step. The identities of the resulting PCR fragments were confirmed by restriction analysis prior to transformation.

Transformation of *B. thailandensis* and *B. pseudomallei*. Cells were prepared for DNA transformation by inoculating DM with individual colonies freshly grown on LB agar (to an optical density at 600 nm of ~0.02 to 0.05). Cultures were grown with shaking at 37°C to an optical density at 600 nm of ~0.5 (typically 3 to 5 h). The cultures were then concentrated approximately 20× by centrifugation (1 min at 15,700 × *g*) and resuspension in DM, and 50-μl aliquots of the concentrated cells were mixed with approximately 100 ng PCR fragment (or chromosomal) DNA in a 5-μl volume. This amount of PCR product was not saturating for transformant yield. The mixture was then incubated without agitation for 30 min at room temperature, followed by the addition of 2 ml DM and incubation for 6 to 48 h at 37°C with agitation (sufficient for about a 200-fold increase in cell number). The cells were then washed with 1 ml DM, resuspended in 250 μl of DM, and plated (typically 100 μl per plate) on LB agar supplemented with appropriate antibiotics to select for growth of recombinants.

Verification of recombinant identity. Candidate mutants resulting from transformation were characterized using two sets of PCRs, one to confirm the presence of the appropriate insertion and a second to confirm the loss of the targeted wild-type sequences. The first set of reactions employed primer pairs in which one primer hybridized outside and the second hybridized inside the PCR fragment used for mutagenesis (primers 4F and 4R and primers 5F and 5R) (Fig. 2A). The generation of amplified fragments of the appropriate size implied that the resistance gene had inserted at the appropriate site in the genome. The second set of reactions examined amplification of the entire genomic region corresponding to the targeted gene (using primers 4F and 5R) (Fig. 2A). The sizes of the fragments generated differed between the wild type and the mutant and thus indicated whether the wild-type gene was absent.

To carry out diagnostic PCRs, individual transformant colonies were suspended in 100 μl water and incubated at 96°C for 10 min. One microliter of the mixture was then used as a template in PCRs with primers 4F and 4R or 5F and 5R (95°C for 2 min [hot start]; 30 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 2 min; and a final extension for 10 min) or with primers 4F and 5R (under the same amplification conditions, except that the extension time ranged from 4.0 to 7.5 min depending on the predicted size of the fragment). The sizes of the products generated were determined using agarose gel electrophoresis.

RESULTS AND DISCUSSION

Targeted mutagenesis of *B. thailandensis* by use of PCR fragments. Based on initial observations that natural transformation could be used to transfer chromosomal insertion mutations between *B. thailandensis* cells (see below), we developed a procedure for targeted mutagenesis by employing PCR fragments. The fragments used for mutagenesis carried tetracycline or trimethoprim resistance markers flanked by chromosomal homology such that integration of the fragments would replace chromosomal loci with the resistance determinants (Fig. 1) (see Materials and Methods). The growth conditions employed to render cells competent were similar to those used with *Ralstonia solanacearum*, a relative of *B. thailandensis* (3). After transformation of the PCR fragments and selection for antibiotic resistance, colonies were screened by analytical PCR to verify that they carried the appropriate insertions. The verification tests examined both insertion of the element at the appropriate genomic site and loss of sequences corresponding to the wild-type sequence (Fig. 2). In practice, it was possible to generate all of 45 *B. thailandensis* mutants attempted, and nearly all (>90%) of the recombinant colonies examined were confirmed to be correct (Table 1 and data not shown). In two cases (Δ*amrRAB* and Δ*gltB* mutants), we also verified that candidate mutants showed expected antibiotic sensitivity and auxotrophic growth phenotypes (not shown).

Several features of the procedure were documented (Table 1). First, mutations could be generated at a number of loci. In our studies, eight different loci were deleted, including genes on both chromosomes. Although it was possible to generate every *B. thailandensis* mutant attempted, the frequencies of mutant recovery varied considerably in independent trials. Second, although both tetracycline resistance and trimethoprim resistance were suitable as selective markers, the recovery of mutants after trimethoprim resistance selection was significantly greater in most experiments. Third, two different strains of *B. thailandensis*, E264 and S95019, were successfully mutagenized. *B. thailandensis* E264 is a reference strain whose genome sequence is currently available (10). Fourth, the re-

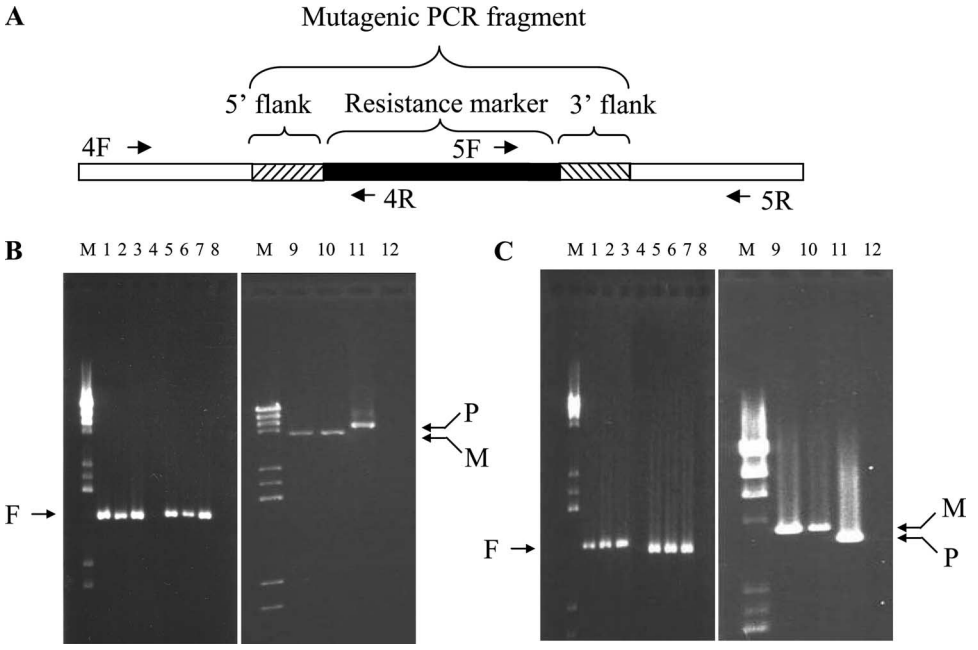

FIG. 2. Mutant verification. (A) Structure of a chromosomal gene replacement generated by incorporation of a mutagenic PCR fragment. Note that primers 4F and 5R fall in genomic regions outside the mutagenic PCR fragment. (B and C) Confirmation of two mutant structures. Colonies from two mutagenesis experiments were subjected to amplification with primers 4F/4R (lanes 1 to 3), 5F/5R (lanes 5 to 7), or (for two of the colonies in each case) 4F/5R (lanes 9 and 10). The predicted migration of fragments corresponding to the 5′ and 3′ ends of the gene replacement is indicated (F), as is that of the full-length mutant (M) and parental (P) fragments. Reaction mixtures lacking cells are shown in lanes 4 (4F/4R), 8 (5F/5R), and 12 (4F/5R), and reactions showing amplification of the parent strain are shown in lanes 11 (4F/5R). Molecular weight markers are shown in lanes M.

covery of recombinants increased with the length of flanking homology (between 400 and 1,500 bp) (Table 1, rows 10 to 14 and 19 to 21). Our standard protocol employs 800-bp homology fragments as a compromise between the needs for efficient generation of recombinants and reliable amplification of the flanking homology fragments by PCR.

We found that it was possible to introduce resistance determinants flanked by site-specific recombinase recognition sequences (*loxP* or *FRT*) and to excise the markers from the genome in a second step by the introduction of plasmids expressing Cre or FLP recombinase (2, 5) (Table 1; see Table S1 in the supplemental material; data not shown). The excision of the resistance determinants makes it possible both to reutilize them for genetic selection and to examine phenotypes without potential complications due to marker effects on physiology.

Transfer of chromosomal mutations between *B. thailandensis* strains. DNA transformation was also used to transfer chromosomal mutations between *B. thailandensis* strains (Table 2). Bacteria grown under the conditions used for PCR fragment mutagenesis were exposed to whole chromosomal DNA preparations, followed by selection for recombinants that had acquired antibiotic resistance-marked mutations. Most of the features associated with PCR fragment mutagenesis were also observed for chromosomal marker transfer, including greater recombinant recovery for crosses in which resistance to trimethoprim rather than tetracycline was selected. Strain S95019 appeared to be transformed more efficiently than E264, in spite of the fact that the donor chromosomal DNA was isolated from E264 mutants. In addition to the transfer of drug resistance markers, it was possible to generate prototrophic recombinants from transformation of an auxotrophic (*gltB* null) mutant with wild-type DNA (not shown). Multiple mutants could also be generated (see Table S1 in the supplemental material).

The frequency of chromosome marker transformation was about 3 orders of magnitude higher than the generation of mutations from PCR products (after normalization for the reduced representation of the mutation in chromosomal DNA relative to the PCR preparations). For example, although the mutant allele typically was present at about 1 copy/2 kbp in the mutagenic PCR preparations and 1 copy/6.7 Mbp in chromosomal DNA, the recovery of recombinants was usually comparable for the two transformations [e.g., compare Table 1, row 8, with Table 2, row 7, for the Δ(*amrRAB*)::*tmp* strain]. We suspect that the enhanced relative recovery of recombinants after chromosomal DNA transformation is due to either the increased homology of the recombining fragments, the absence of restriction acting on the DNA, or both factors. In practice, it would appear preferable to generate new strains by transferring mutations from preexisting strains by using chromosomal DNA rather than creating them newly using PCR fragments in order to eliminate the risk of introducing allelic differences due to sequence heterogeneity in PCR preparations.

Genetic manipulation of *B. pseudomallei*. We found that both the PCR mutagenesis and genetic transfer procedures worked out with *B. thailandensis* could be extended to *B. pseudomallei* strains 1026a and 1026b (Table 3). The frequencies at which recombinants were recovered were reduced relative to those for the *B. thailandensis* strains in both processes,

TABLE 1. Generation of *B. thailandensis* deletion mutants by use of PCR fragments

Locus[a]	Gene product[a]	Recipient strain	Marker (size [bp])[b]	Transformation efficiency (no. of colonies/ng/10^9 cells)		No. of confirmed transformants/no. of transformants examined[c]
				Avg	Range[d]	
gltB (BTH_I3014)	Glutamate synthase	E264	*tet* (800)	2.2	1.1–2.2 (3)	2/2
BTH_I1592	Outer membrane porin protein	E264	*tet* (800)	6.6	1.1–21 (6)	50/55
BTH_I2740	Type I restriction endonuclease	E264	*tet* (800)	2.2	2.2 (2)	19/20
BTH_I2740	Type I restriction endonuclease	E264	*loxP-tet-loxP* (800)	0.3	0.2–0.4 (3)	8/8
BTH_I0042	Type III restriction endonuclease	E264	*tet* (800)	2.2	1.1–3.3 (3)	6/8
mutS (BTH_I1913)	Mismatch repair protein	E264	*tet* (800)	1.1	0.4–2.2 (2)	8/8
mutS (BTH_I1913)	Mismatch repair protein	E264	*FRT-tmp-FRT* (800)	160	160 (1)	2/2
amrRAB (BTH_I2444-6)	Multidrug efflux pump	E264	*tmp* (800)	26	11–45 (6)	10/10
envZ-ompR (BTH_I0893-4)	Two-component regulator	E264	*tmp* (800)	180	24–440 (9)	12/14
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	E264	*tmp* (400)	19	11–27 (2)	2/2
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	E264	*tmp* (800)	140	41–220 (4)	2/2
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	E264	*tmp* (1,500)	420	400–440 (2)	2/2
mutS (BTH_I1913)	Mismatch repair protein	E264	*loxP-tmp-loxP* (800)	7.7	5.5–11 (3)	1/2
mutS (BTH_I1913)	Mismatch repair protein	E264	*loxP-tmp-loxP* (1,500)	11	11 (1)	4/4
gltB (BTH_I3014)	Glutamate synthase	S95019	*tet* (800)	37	12–69 (3)	
BTH_I1592	Outer membrane porin protein	S95019	*tet* (800)	1.6	1.6 (1)	
amrRAB (BTH_I2444-6)	Multidrug efflux pump	S95019	*tmp* (800)	6.8	23–170 (4)	
envZ-ompR (BTH_I0893-4)	Two-component regulator	S95019	*tmp* (800)	170	130–280 (5)	
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	S95019	*tmp* (400)	13	13 (1)	2/2
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	S95019	*tmp* (800)	65	19–110 (2)	2/2
bpeEF-oprC (BTH_II2104-6)	Multidrug efflux pump	S95019	*tmp* (1,500)	1,630	1,630 (1)	2/2

[a] Obtained from the TIGR Comprehensive Microbial Resource database (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gbt).
[b] *tet*, tetracycline resistance; *tmp*, trimethoprim resistance.
[c] Transformants were verified/examined by PCR for each mutagenesis experiment (Fig. 2).
[d] Values in parentheses correspond to the number of independent trials.

but it was still possible to generate the majority of mutants and chromosomal recombinants sought (Table 3 and data not shown). Repeated attempts to generate targeted mutations by PCR fragment transformation in *B. pseudomallei* strain K96243 (whose genome sequence is available) were unsuccessful (data not shown).

TABLE 2. Genetic transfer of *B. thailandensis* chromosomal insertions

Donor DNA[a]	Recipient strain	Transformation efficiency (no. of colonies/ng/10^9 cells)	
		Avg	Range
gltB::IS*lacZ*/hah	E264	12	3.3–32
BTH_I1592::IS*lacZ*/hah	E264	22	4.4–54
Δ(*gltB*)::*tet*	E264	5.5	2.2–9.9
Δ(*mutS*)::*tet*	E264	2.2	2.2
Δ(*mutS*)::*loxP-tmp-loxP*	E264	20	13–26
Δ(*envZ-ompR*)::*tmp*	E264	13	1.1–32
Δ(*amrRAB*)::*tmp*	E264	20	5.5–38
Δ(*bpeEF-oprC*)::*tmp*	E264	36	36
Δ(*gltB*)::*tet*	S95019	190	76–330
Δ(*mutS*)::*tet*	S95019	10	10
Δ(*mutS*)::*loxP-tmp-loxP*	S95019	39	39[b]
Δ(*envZ-ompR*)::*tmp*	S95019	160	80–230
Δ(*amrRAB*)::*tmp*	S95019	600	310–970
Δ(*bpeEF-oprC*)::*tmp*	S95019	640	640

[a] Chromosomal DNA was isolated from *B. thailandensis* strain E264 carrying the indicated mutations.
[b] Three of three transformants were verified by PCR (Fig. 2).

Conclusions. The targeted mutagenesis procedure described here is based on the natural transformation of PCR fragments carrying mutations whose recombination into the genome can be selected. Transformation may be mediated by type IV pilus assembly and disassembly systems (1), which are encoded in both *Burkholderia* genomes (8, 10). Mutagenesis was used to generate 45 *B. thailandensis* and 16 *B. pseudomallei* deletion strains, nearly all of those attempted, with fewer than 10% false-positive results. Although the new procedure requires that a selectable marker be present in the integrating fragment, it is possible to eliminate the marker by site-specific recombination if an appropriate element is employed. In addition to generating chromosomal deletions, it should be straightforward to use these procedures to introduce new sequences without a loss of resident genetic material.

Although it is currently not permissible in the United States under CDC regulations to introduce tetracycline or trimethoprim resistance into *B. pseudomallei*, CDC-compliant selective markers have been developed (5). The techniques described here should thus be globally useful if such markers are employed.

Natural transformation of whole chromosomal DNA was also employed to move mutations between strains. This genetic transfer method should facilitate strain construction (e.g., of multiple mutants for epistasis studies), recombination-based genetic mapping, and verification of linkage of constructed mutations and the traits they confer.

In summary, this report describes simple procedures for

TABLE 3. Targeted mutagenesis and mutation transfer in *B. pseudomallei*

Mutant	Recipient strain	Marker (length of flanking homology [bp])[a]	Transformation efficiency (no. of colonies/ng/10^9 cells)		No. of confirmed transformants/no. of transformants examined[b]
			Avg	Range	
Mutants created by PCR fragment mutagenesis					
Δ(*mutS*)	1026b	*FRT-tmp-FRT* (800)	6.1	6.1	2/2
Δ(*amrRAB*)	1026b	*tmp* (800)	2.4	0.2–6.1	
Δ(*bpeEF-oprC*)	1026b	*tmp* (1,500)	4.9	0.6–8.5	6/6
Δ(*amrRAB*)	1026a	*tmp* (800)	1.7	0.6–2.3	
Δ(*mutS*)	1026a	*loxP-tmp-loxP* (1,500)	1.2	0.3–2.3	4/4
Mutants created by chromosomal DNA transfer					
Δ(*amrRAB*)	1026b	*tmp*	23	1.2–57	
Δ(*bpeEF-oprC*)	1026b	*tmp*	6.1	3.0–9.2	1/2
Δ(*mutS*)	1026b	*loxP-tmp-loxP*	51	51	4/4
Δ(*amrRAB*)	1026a	*tmp*	17	4.1–30	
Δ(*bpeEF-oprC*)	1026a	*tmp*	9.3	9.3	3/4

[a] *tet*, tetracycline resistance; *tmp*, trimethoprim resistance; *loxP*, recognition site of Cre recombinase; *FRT*, recognition site of FLP recombinase.
[b] Colonies were verified/examined by PCR tests to determine the generation of the appropriate mutation and the loss of wild-type sequences.

generating targeted mutations and for genetic transfer of chromosomal markers in *B. thailandensis* and *B. pseudomallei*. The new procedures should facilitate functional studies of genes identified from the genome sequences of the two species.

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