Sequence-Defined Transposon Mutant Library of Burkholderia thailandensis

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ABSTRACT We constructed a near-saturation transposon mutant library for Burkholderia thailandensis, a low-virulence surrogate for the causative agent of melioidosis (Burkholderia pseudomallei). A primary set of nearly 42,000 unique mutants (~7.5 mutants/gene) was generated using transposon Tn5 derivatives. The strains carry insertions in 87% of the predicted protein-coding genes of the organism, corresponding to nearly all of those nonessential for growth on nutrient agar. To achieve high genome coverage, we developed procedures for efficient sequence identification of insertions in extremely GC-rich regions of DNA. To facilitate strain distribution, we created a secondary library with two mutants per gene for which most transposon locations had been confirmed by resequencing. A map of mutations in the two-allele library and procedures for obtaining strains can be found at http://tools.nwrce.org/tn_mutants/ and http://www.gs.washington.edu/labs/manoil/. The library should facilitate comprehensive mutant screens and serve as a source of strains to test predicted genotype-phenotype associations.

IMPORTANCE The Gram-negative bacterium Burkholderia pseudomallei is a bioterror threat agent due to its potential for aerosol delivery and intrinsic antibiotic resistance and because exposure produces pernicious infections. Large-scale studies of B. pseudomallei are limited by the fact that the organism must be manipulated under biological safety level 3 conditions. A close relative of B. pseudomallei called Burkholderia thailandensis, which can be studied under less restrictive conditions, has been validated as a low-virulence surrogate in studies of virulence, antibiotic resistance and other traits. To facilitate large-scale studies of B. thailandensis, we created a near-saturation, sequence-defined transposon mutant library of the organism. The library facilitates genetic studies that identify genotype-phenotype associations conserved in B. pseudomallei.

The Gram-negative bacterium Burkholderia thailandensis is a close relative of Burkholderia pseudomallei, which is the causative agent of melioidosis and a Tier 1 select agent (1, 2). Both species are found in the soil and have large genomes with two chromosomes and high levels of gene synteny and sequence conservation (3). B. thailandensis is not normally virulent toward humans, but at high doses it can infect rodents, causing a melioidosis-like disease (4, 5). Like B. pseudomallei, B. thailandensis infects mammalian tissue culture cells by escaping phagocytic vacuoles, moving through the cytoplasm, inducing giant cell formation, and spreading to adjacent cells (6–9). In addition, B. thailandensis infects insects, nematodes, plants, and slime molds, often exceeding B. pseudomallei in virulence (10–15). B. thailandensis encodes homologues of many known B. pseudomallei virulence functions, including type III secretion, type VI secretion, and quorum-sensing systems (16, 17). Both species also encode a remarkable variety of functions that inhibit growth of competing microbes, including contact-dependent growth inhibition systems, type VI effectors, antibiotics, and other small molecules (17–23). Both B. pseudomallei and B. thailandensis are also intrinsically resistant to several antibiotics due to the actions of orthologous efflux pumps (24, 25). There are likely many additional similarities between the two organisms yet to be discovered. Because it may be manipulated under less restrictive laboratory conditions, B. thailandensis represents an attractive surrogate for B. pseudomallei.

Two B. pseudomallei auxotrophic mutants exempt from the select agent rule have recently become available (26, 27). The strains are highly attenuated in their virulence and promise to be highly useful for studying processes other than whole animal infection.

Several procedures facilitate genetic manipulation of B. thailandensis and B. pseudomallei. Natural transformation can be employed for targeted mutagenesis using PCR fragments or to transfer mutations between strains (25, 28). Procedures for transposon mutagenesis and complementation have also been developed (29–31).

Genetic analysis of several bacterial species has been accelerated by the availability of comprehensive sequence-defined mutant libraries (32–35). Such libraries provide a resource for relatively complete mutant screens as well as directed tests of the functions of specific gene products (36). In this report, we describe the construction of a comprehensive transposon mutant library for B. thailandensis.
RESULTS AND DISCUSSION

Overview. We sought to create a comprehensive sequence-defined transposon mutant library of *B. thailandensis*. To achieve this goal, we first generated and sequence mapped a near-saturation collection of “random” insertion mutants. To reduce the size of the library while retaining genome coverage, a subset of strains corresponding to approximately two mutations per gene were colony purified and rearrayed. Mutants of this two-allele library were then resequenced to verify insertion assignments.

Mutant isolation and sequencing. Mutants of *B. thailandensis* E264 were generated using two transposon Tn5 derivatives (Fig. 1). More than 64,000 such mutants were initially arrayed, and their transposon-genome junction regions were amplified using a thermostable DNA polymerase (TSG). Junction fragments were Sanger sequenced, and insertion sites were mapped to the genome (see Materials and Methods) (37). This round of sequencing identified 33,358 unique strains (~6 unique mutants per gene), corresponding to insertions in about 80% (4,492) of the organism’s 5,634 predicted coding genes.

This level of genome coverage was lower than expected from previous mutant library construction projects by 5 to 10% (data not shown) (37). The apparent explanation for the low coverage was inefficient identification of transposon insertions in exceptionally high-GC regions of the genome (Fig. 2A and B). The inefficient identification of insertions in very-high-GC regions was also seen in a transposon sequencing (Tn-seq) assay of a large (530,000 members) pool of T23 insertion mutants (Fig. 2C). The pattern of reads produced by the Tn-seq assay was revealing: although insertions in very-high-GC regions were observed, the average numbers of sequence reads for these insertions were low (e.g., compare BTH_12705 to BTH_12701 in Fig. 2C). The results suggested that although insertions in the very-high-GC regions could be generated, their identification by sequencing was inefficient.

We made two changes to improve the detection of insertions in very-high-GC regions of the genome. First, we increased the GC content of the primers used to amplify transposon-genome junction fragments for sequencing (see Text S1 in the supplemental material). Second, we carried out the amplification of junction fragments with a different polymerase (KAPA) that acts efficiently on GC-rich sequences (38). We then used the new procedure to resequence arrayed mutants that had failed to be successfully sequenced when TSG polymerase was used in the initial runs. We found that many of the insertions could now be mapped and that many were indeed in the underrepresented GC-rich regions (Fig. 2B and D). Ultimately, we resequenced 9,759 mutants that could not be mapped in the initial sequencings and identified insertions in 336 additional genes. The final primary defined mutant collection provided 86.7% coverage of predicted genes (4,886/5,634) (Table 1). Genes unrepresented in the library correspond mainly to candidate essential genes (39).

Two-allele mutant library. To create a smaller library of mutants providing genome coverage, we assembled a set of two unique mutants for genes represented in the primary mutant collection. We included two rather than one insertion per gene to reduce missed genotype-phenotype associations due to cross-contamination and other issues and to provide confirmation of associations. The two-allele library strains corresponded where possible to insertions situated between 5% and 85% of the coding sequence and at a distance from one another within each gene (Table 2). Most of the mutations (84%) were transposon T23 insertions. We confirmed the identities of 81% of the two-allele set by resequencing (Table 2).

The 12,322 mutants making up the two-allele library are listed in Table S1 in the supplemental material. The table includes sequence quality metrics for each strain and indicates which mutants were confirmed by resequencing. A map of two-allele library insertions relative to the annotated *B. thailandensis* genome is provided at http://tools.nwrce.org/tn_mutants/ (Fig. 3).

Deletion of transposon sequences by recombination. We verified the functionality of the transposon T23 FRT recombination target (FRT) sites in *B. thailandensis*. Recombination at the sites is predicted to leave a short sequence in which nearly all internal transposon sequences have been lost (Fig. 4A). The recombination was demonstrated by transiently introducing a plasmid expressing FLP recombinase into three T23 mutants to induce recombination. PCR assays showed that putative recombinant colonies had indeed lost transposon sequences and carried short insertions of DNA in their place (Fig. 4B). In addition, as expected, the corresponding recombinants had lost β-galactosidase activity in two cases in which the corresponding T28 insertions had generated active lacZ gene fusions and had lost trimethoprim resistance for all three strains (Fig. 4C).
Uses of the two-allele mutant library. The defined mutant library should facilitate genetic analysis of *B. thailandensis* in several ways.

- The library can be screened for strains exhibiting changes in phenotypes of interest to provide relatively complete lists of nonessential genes involved in the corresponding processes (34). Carrying out the process for multiple phenotypes can refine genome annotations and lead to discoveries (36, 40).
- The library provides a source of mutants to test hypothesized genotype-phenotype associations. For example, the *in vivo* roles of genes predicted to be involved in different processes experimentally or through bioinformatics analysis have been tested using corresponding mutants from the library (41, 42; L. A. Gallagher, unpublished results).
- Since the transposons used to generate the mutant library can generate β-galactosidase reporter gene fusions, they can be used to monitor expression of the corresponding target genes (43).
- Since the transposon used to generate most of the mutants also carries a regulated outward-facing rhamnose promoter at one end, it can be used to provide regulated expression of genes adjacent to insertion sites (44, 45).
Since the transposons also carry the FRT gene or loxP sites at their ends, resistance determinants of the transposons can be deleted by site-specific recombination. The process can facilitate the construction of multiple mutants by an iterative transformation procedure (46).

A possible concern with the library arises from the fact that the E. coli donor strain used for transposon mutagenesis (SM10/Apir) carries phage Mu DNA that can potentially be transferred by conjugation to recipient B. thailandensis strains during T8 or T23 mutagenesis (47). Such transfer could lead to Mu insertions in transposon mutant strains. While such insertions appear to be rare (a PCR assay of 66 strains chosen randomly from the two-allele mutant library detected Mu sequences in only one strain [data not shown]), it is nonetheless particularly important to verify that phenotypes found for strains of the library are due to the T8 or T23 insertions rather than secondary mutations. We would thus recommend that genotype-phenotype associations be confirmed for both insertion alleles of a gene represented in the two-allele library and/or that genetic linkage be examined by natural transformation of transposon insertion alleles into the “clean” parent background (25) followed by phenotype retesting.

We believe that the defined mutant library should facilitate a variety of genome-scale genetic studies of B. thailandensis. Such studies should help reveal the functional basis of important traits shared by B. pseudomallei, as well as provide a better understanding of how the two species differ from each other. Strains from the two-allele library may be obtained by following instructions at http://www.gs.washington.edu/labs/manoil/thailandensis_library.htm. The Transposon Mutant Library Browser (http://tools.nwrce.org/tm_mutants/) provides a simple graphical interface to facilitate identifying and requesting strains.

### MATERIALS AND METHODS

**Strains, media, and growth conditions.** The transposon mutant library was constructed using B. thailandensis E264 (48). Transposon T8 (ISlacZ/ hah-tc) and its conjugal delivery suicide plasmid (pIT2) have been described previously (37). Transposon T23 (ISlacZ-Pyhalotin-tp/FRT) was carried on conjugal delivery plasmid pLG99 (see Fig. S1 in the supplemental material). The growth media were LB broth (10 g tryptone, 5 g yeast extract, and 8 g NaCl per liter), tryptone-yeast extract (TYE) agar (LB broth with 1.5% agar), tryptic soy broth (TSB; Difco 211825), tryptic soy agar (TSA; Difco 2360950), 2× nutrient TSA (TSA with an additional 30 g/liter TSB powder), TSB freezer medium (TSB with 10% [wt/vol] glycerol), and M9 minimal medium supplemented with 1 μg/ml vitamin B1 and 0.2% or 0.4% pyruvate. For T23 lacZ reporter activity and FRT recombination tests, TYE medium was supplemented with 40 μg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) or 50 μg/ml trimethoprim. Plasmids pIT2 and pLG69 were maintained in Escherichia coli using TYE containing 100 μg/ml carbenicillin.

**Mutagenesis and library construction.** Mutagenesis was carried out using methods similar to those used previously (37). Specifically, overnight cultures of E. coli donor strain SM10/Apir (49) carrying pIT2 or pLG99 and recipient strain B. thailandensis E264 were subcultured (1:50

#### TABLE 2  B. thailandensis two-allele mutant library

| Parameter                          | No.  |
|------------------------------------|------|
| Total mutants                      | 12,322|
| Within genes                       | 11,510|
| Intergenic                         | 812   |
| Genes with insertions              |       |
| Total                              | 4,667 |
| 1 unique insertion                 | 1,033 |
| 2 unique insertions                | 1,480 |
| 3 unique insertions                | 1,449 |
| 4 unique insertions                | 507   |
| >4 unique insertions               | 198   |
| Genes without insertions           | 964   |
| Transposon types                   |       |
| T8 insertions                      | 1,982 |
| T23 insertions                     | 10,340|
| Mutants confirmed by resequencing  | 9,247 |

* A total of 11,407 of the 12,322 strains were successfully resequenced at least once. Mutant assignments were considered confirmed if the resequencing matched the initial assignment (8,882 strains) or if multiple resequencings confirmed a new location (365 strains). Strains for which a single resequencing indicated a new location (1,949 strains), unless the resequencing was of poor quality, in which case the original assignment was kept (211 strains).

#### FIG 3  Web browser representation of two-allele library insertion mutations. A partial screen shot of the transposon locations in a representative region of the B. thailandensis genome provided at http://tools.nwrce.org/tm_mutants/ is shown. Transposons are represented as triangles, with positions above or below the line corresponding to their orientations in the genome. Filled triangles represent sequence-confirmed insertions, and open triangles represent unconfirmed insertions.
for the donor and 1:10 for the recipient) and grown for approximately
90 min on a 37°C roller. Equal volumes (0.5 to 3.0 ml) of donor and
recipient subcultures were then mixed and centrifuged, the pellet resus-
pended in 20 ml of LB with 20 mM MgSO₄, spotted onto a nitrocellulose
filter (0.45-μm pore size) on prewarmed TYE agar, and incubated for
approximately 2 h at 37°C. Cells were washed from the filter and plated on
TYE agar containing streptomycin (100 μg/ml) and either tetracycline
(60 μg/ml) for pIT2 mutagenesis or trimethoprim (50 μg/ml) for pLG99
mutagenesis. After incubation for 2 days at 37°C, resistant colonies were
picked and arrayed into 384-well plates containing TSB freezer medium
using a QPix2 colony picking robot (Genetix). Plates were incubated for
24 h at 37°C and 16 h at room temperature and then stored at −80°C.

**Mutant identification.** Insertion sites were identified by semidegen-
erate PCR and sequencing of the transposon-genome junctions (37, 50)
(see Text S1 in the supplemental material).

**Tn-seq.** Approximately 530,000 T23 insertion mutants were pooled
(pool “BtL1”), and genomic DNA was isolated from an aliquot of the
pool. The Tn-seq circle method (51) with minor modifications was used
to identify the transposon insertion locations for the pooled mutants (see
Text S1 in the supplemental material).

**FLP recombination of T23 insertion mutants.** FLP-mediated marker
excision of T23 insertion mutants was carried out using plasmid pFLPe4
(29). Excision was verified by colony PCR using a transposon-specific
primer and locus-specific primers flanking the site of insertion. The oli-
gonucleotides used are listed in Text S1 in the supplemental material.

**Two-allele mutant set.** A Perl script and manual curation were used to
choose the two mutants for each gene that best combined four criteria: (i)
insertions situated between 5% and 85% of the coding sequence of the
gene, (ii) insertions situated at substantial distance from one another
within the 5 to 85% window (with the hits selected spaced at least 100 bp

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**FIG 4** Transposon T23 internal sequences can be excised by FLP recombination. (A) The deletion of transposon sequences by FLP recombination at the FRT
sites in T23 is illustrated, along with PCR primers used for analysis. The sequence of the 96-bp insertion left after recombination is CTGTCTATACAT
CTAAGATCCTATCTCCGAATTCCTATTCTTAGAAAACTTCCGTCGGCAGATGTGTATAAGAGACAG (not including the 9 bp of
target sequence duplicated at each insertion site). (B) Analysis of insertion sites by PCR. For three T23 insertions examined, the wild-type (E264) PCR fragment
(+F + R) disappeared in the corresponding transposon insertion mutant and was replaced by a slightly larger fragment in the FRT-FRT recombinant. The presence
of the transposon in each case was verified by PCR using a transposon-specific primer (F + Tn). (C) Phenotypic tests of recombinants. Two of the T23 insertions
analyzed carried ‘lacZ in the same orientation as the chromosomal target gene (denoted as “+”) and generated LacZ+ cells, which were blue on X-Gal
(5-bromo-4-chloro-3-indolyl galactopyranoside) medium (top). The β-galactosidase activity was eliminated by FRT-FRT recombination, as expected. Tp,
trimethoprim.
apart for more than 85% of the genes and at least 200 bp apart for greater than 75% of the genes), (iii) sequence mapping data of high quality, and (iv) T23 insertion mutants favored over T8 insertion mutants. To assemble the set, strains from the original library were cherry-picked using the QPix2 robot into 96-well plates (Genetix X6011) containing 180 μl of TSB per well. After picking, the plates were covered with a sterile plastic lid and grown 4 h at 37°C in a shaking incubator at 200 rpm. Three serial dilutions were then made by transfer of ~5–μl aliquots of culture per well into new 96-well plates containing 60 μl of TSB per well using a grooved 96-pin replicator. The grooved pin replicator was then used to transfer ~5 μl of medium per well from each of the dilution plates onto 2× nutrient TSA medium with 100 μg/ml streptomycin and 50 μg/ml tetracycline or trimethoprim, depending on the transposon. The stamped dilution plates were grown overnight at 37°C, and an isolated single colony of each strain was picked into a 96-well, deep well block containing 1.2 ml of TSB freezer medium per well. The blocks were covered with Airpore tape sheets (Qiagen, 19571) and grown overnight at 37°C with shaking. After growth, aliquots of the culture were distributed into single-tube racks (Matrix TrakMates 3735) before freezing.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00604-13/-/DCSupplemental.

ACKNOWLEDGMENT

This work was supported by grant U54AI051741 from the National Institutes of Health.

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