An Improved Method for *oriT*-Directed Cloning and Functionalization of Large Bacterial Genomic Regions

Brian H. Kvitko,* Ian A. McMillan,* Herbert P. Schweizer

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, USA

We have made significant improvements to a broad-host-range system for the cloning and manipulation of large bacterial genomic regions based on site-specific recombination between directly repeated *oriT* sites during conjugation. Using two suicide capture vectors carrying flanking homology regions, *oriT* sites are recombined on either side of the target region. Using a broad-host-range conjugation helper plasmid, the region between the *oriT* sites is conjugated into an *Escherichia coli* recipient strain, where it is circularized and maintained as a chimeric mini-F vector. The cloned target region is functionalized in multiple ways to accommodate downstream manipulation. The target region is flanked with Gateway *attB* sites for recombination into other vectors and by rare 18-bp I-SceI restriction sites for subcloning. The Tn7-functionalized target can also be inserted at a naturally occurring chromosomal *attTn7* site(s) or maintained as a broad-host-range plasmid for complementation or heterologous expression studies. We have used the *oriTn7* capture technique to clone and complement *Burkholderia pseudomallei* genomic regions up to 140 kb in size and have created isogenic *Burkholderia* strains with various combinations of genomic islands. We believe this system will greatly aid the cloning and genetic analysis of genomic islands, biosynthetic gene clusters, and large open reading frames.

To aid the genetic characterization of strain-to-strain genomic variation and the heterologous production of natural products, the genome-scale manipulations required by synthetic biology necessitate the specific cloning and manipulation of DNA regions greater than 10 kb (1–4). However, PCR amplification- and restriction fragment-based cloning techniques are inefficient, inaccurate, and not always possible for regions of >10 kb. Several techniques have been developed to bridge this technical gap. The *oriT*-directed capture system developed by Chain et al. has advantages for the specific cloning of large genomic regions compared with site-specific recombinase-catalyzed “pop-out” and short-flank recombineering-based strategies (2, 5–10). The system is based on the principle that recombination will occur between two directly oriented RP4 origin of transfer (*oriT*) sites during conjugative transfer (11). The *oriT*-directed capture system utilizes two suicide vectors, one of which is mini-F replicon based, each with different selectable markers. Segments flanking the target region are cloned into the suicide vectors, which are then homologously recombined into the donor strain genome, resulting in directly oriented *oriT* sites flanking the target region. After transformation of this target donor strain with a conjugation helper plasmid, the conjugation machinery encoded by this plasmid recognizes one of the *oriT* sites integrated into the genome and begins transfer in a directional manner of downstream genomic DNA, including the target region and the mini-F replicon, into an *Escherichia coli* recipient. Transfer is terminated upon reaching the second *oriT* site, which is then recombined with the initiating *oriT* in the *E. coli* recipient, thus generating a circular mini-F-based plasmid carrying the target genome region. The system developed by Chain et al. (5) was used to clone specific regions in excess of 200 kb and does not rely on the location of flanking restriction sites required by RecE-based recombineering strategies (2, 5). The *oriT*-directed capture system does not generate deletions in the target chromosome during capture, because only one strand of the genome is mobilized. This prevents any fitness effects that might be caused by large deletions seen in site-specific recombinase-based pop-out

systems (10). The circularized capture vector is simultaneously generated and recovered as an *E. coli* transconjugant. This eliminates the need for organism-specific conditional replicons, additional plasmid rescue steps, and the potential difficulties associated with the recovery of large plasmids. The captured DNA is recovered directly from the donor genome, which eliminates the introduction of point mutations. Finally, all steps involving the handling of large DNA are conducted *in vivo*, which reduces issues of large fragment DNA instability and large vector transformation inefficiency. This system should be functional in any organism that can be established as a conjugation donor.

We have engineered significant improvements into the *oriT*-directed capture system for increased functionality and the downstream manipulation of captured target regions. Most notably, we created new broad-host-range conjugation helpers and have incorporated mini-Tn7 functionalization for single-copy genomic integration to create the *oriTn7* capture system. The transposon Tn7 is capable of integrating site-specifically into a naturally evolved *attTn7* genomic site located downstream of highly conserved *glmS* genes (12–14). The final pFTarget vector incorporates portions from each capture vector so that the large target region is carried by a complete mini-Tn7 transposon. We have built the

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Address correspondence to Herbert P. Schweizer, Herbert.Schweizer@colostate.edu.

* Present address: Brian H. Kvitko, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA; Ian A. McMillan, Department of Molecular Bioscience and Bioengineering, University of Hawaii at Manoa, Honolulu, Hawaii, USA.

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oriTn7 capture system for use in *Burkholderia pseudomallei*, the causative agent of melioidosis and a category B select agent. There is extensive strain-to-strain variation within the *B. pseudomallei* pangenome, much of this in genomic regions of difference greater than 10 kb, and many of these regions are genomic islands (15–21). The characterization of natural gene loss, gene acquisition, and strain-to-strain variability is likely to be important in understanding *B. pseudomallei* pathogenicity and phenotypic diversity. This characterization requires the creation of isogenic strains carrying specific deletions or insertions of the genomic regions of difference. We have successfully used the oriTn7 capture system to specifically clone genomic regions from 12 kb to 140 kb corresponding to a region absent from the human clinical *B. pseudomallei* isolate 708a and have used Tn7 integration to complement the matching 140-kb deletion of this region. In addition, we have used oriTn7 capture to create a set of isogenic *Burkholderia* strains carrying various combinations of two *B. pseudomallei* gene clusters whose presence is geographically biased in strains from different regions where this pathogen is endemic. Although in its current form, the oriTn7 capture system was built specifically for use in *B. pseudomallei* and related species, it was designed with portability in mind and could be readily adapted for use in other bacteria.

MATERIALS AND METHODS

Media and culture conditions. Strains used in this study are listed in Table S1 in the supplemental material. Bacteria were cultured in liquid or on agar-solidified Lennox LB (Mo Bio Laboratories, Carlsbad, CA) at 37°C with aeration. *Burkholderia* strains carrying temperature-sensitive plasmids were maintained at 30°C, and plasmid curing was achieved by incubation at 42°C in the absence of selection. All procedures involving *B. pseudomallei* were performed in approved select agent biosafety level 3 (BSL3) facilities in the Rocky Mountain Regional Biosafety Laboratory (CSU) using approved procedures and protocols compliant with regulations regarding select agents. For *E. coli* cultures, media were supplemented with antibiotics at the following final concentrations: ampicillin (Amp), 100 µg/ml; kanamycin (Km), 40 µg/ml; zeocin (Zeo), 25 µg/ml; trimethoprim (Tmp), 100 µg/ml; chloramphenicol (Cm), 25 µg/ml; streptomycin (Sm), 50 µg/ml; gentamicin (Gm), 10 µg/ml; rifampin (Rf), 50 µg/ml; polymyxin B (PmB), 15 µg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), 40 µg/ml; and diamminopimelic acid (DAP), 200 to 400 µg/ml. For *Pseudomonas syringae* cultures, media were supplemented with Rf at a final concentration of 50 µg/ml. For culture of *Burkholderia thailandensis*, media were supplemented with antibiotics at the following final concentrations: Km, 40 µg/ml; Zeo, 25 µg/ml; Gm, 10 µg/ml; and trimethoprim (Tmp), 100 µg/ml. For *B. thailandensis* AmrAB-OprA efflux pump-expressing strains, antibiotic concentrations were adjusted to 500 to 1,000 µg/ml Km and 1,000 to 2,000 µg/ml Zeo. For *B. pseudomallei*, concentrations were as follows: Km, 40 µg/ml; Zeo, 25 µg/ml; Gm, 10 µg/ml; and PmB, 15 µg/ml. For *B. pseudomallei* AmrAB-OprA efflux pump-expressing strains, antibiotic concentrations were adjusted to 500 to 1,000 µg/ml Km and 1,000 to 2,000 µg/ml Zeo. Counterselection of *B. pseudomallei* was done with Tmp at 100 µg/ml and Rf at 50 µg/ml. Arabinose (ARA) or rhamnose (RHA) was used at a final concentration of 0.2% to induce gene expression from plasmids contained in *Burkholderia* spp.

DNA manipulation. Plasmid DNA was purified using the GeneJET plasmid miniprep kit (Fermentas, Glen Burnie, MD). Cleanup of DNA enzymatic reactions and gel extractions were conducted using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis, MO). Bacterial genomic DNA was isolated using the Puregene genomic DNA purification kit (Gentra Systems, Qiagen, Valencia, CA). DNA boiling preparations for

PCR analysis were obtained by combining 1 µl of overnight culture with 30 µl distilled water (dH₂O) or picking a bacterial colony or patch with a sterile toothpick followed by suspension in 30 µl dH₂O and incubation at 100°C for 10 min. Site-directed mutagenesis was conducted with the QuikChange site-directed mutagenesis kit according to the manufacturer's recommendations (Stratagene, Agilent Technologies, Santa Clara, CA). DNA restriction endonucleases, T4 ligase, calf alkaline intestinal phosphatase, and *Taq* polymerase were obtained from New England BioLabs (Ipswich, MA) and used according to the manufacturer's recommendations. HiLo and λ DNA monoclut mix DNA ladders were obtained from Minnesota Molecular (Minneapolis, MN) and New England BioLabs, respectively. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table S2 in the supplemental material. DNA sequencing was conducted using an ABI 3130XL genetic analyzer (Applied Biosystems, Carlsbad, CA) at the Colorado State University Proteomics and Metabolomics Facility. For multiplex PCR, oligonucleotide primers for each probe were designed to have annealing temperature differences of 1°C and amplicon size differences of 100 bp. Multiplex oligonucleotides were combined into a master mix and used at a 4 to 12 µM final concentration. Southern blot analysis was performed using the NEBlot Phototope and Phototope-Star chemiluminescent labeling and detection kits from New England BioLabs following the manufacturer's recommendations and using standard capillary transfer and blotting procedures (22).

Plasmid transformation of *E. coli* was done by using either standard electroporation or chemical transformation procedures (22). Electrotransformation of *B. pseudomallei* was conducted as described previously (23). Briefly, overnight cultures were harvested by centrifugation, washed three times with 300 mM sterile sucrose, and concentrated 10-fold prior to electroporation with 0.2 to 1.0 µg plasmid DNA.

Bacterial conjugations were conducted as either biparental or multiparental matings with RHO3 (or derivative) conjugation donors or pRK2013 (or derivative) conjugation helper plasmids. One to three ml overnight cultures was harvested by centrifugation, washed twice with fresh LB, and concentrated, typically 5-fold, to 10⁹ to 10¹⁰ CFU/ml. Equal parts of each parent strain were combined and applied to sterile cellulose acetate or nitrocellulose filters placed on LB plates (augmented with DAP for conjugations including RHO3 donor strains) with each parent strain spotted individually as a control. Spots were allowed to dry, and plates were incubated overnight at 37°C unless otherwise noted. Cells were recovered from the filters by centrifugation in 1.5-ml microcentrifuge tubes with 1 ml of LB, washed with 1 ml LB, and spread onto LB plates with appropriate selection and counterselection to recover the desired exconjugants.

Plasmid and strain construction. Plasmids used in this study are listed in Table S3 in the supplemental material. Plasmid and strain construction details are provided in the supplemental methods.

OriTn7 capture. (i) **Capture vector LR Clonase recombination.** Left and right flanking regions of homology to target regions were amplified by PCR and cloned into directional pENTR vectors (see the supplemental methods). LR recombination between the right flanking fragment containing pENTR vectors and the pUCTCAPR-GW right capture vectors was facilitated by the compatible Gm^r and Zeo^r markers carried by pUCTCAPR. The pENTR vectors carrying various right flanking fragments were LR recombined with pUCTCAPR vectors using either LR Clonase or LR Clonase II from Invitrogen Life Technologies according to the manufacturer's recommendations. LR recombinants were recovered on LB+Gm plates, screened for Zeo^r, and confirmed by NotI and/or BsrGI restriction enzyme digests.

The design of the left capture vector pFTCAPL-GW required the incorporation of a Km^r resistance marker to comply with select agent regulations for *B. pseudomallei*. Unfortunately, this conflicts with the Km^r marker carried by directional pENTR/D-TOPO vectors. Two strategies were used to overcome the Km^r marker conflict between the pENTR vectors and pFTCAPL vector. In the first strategy, pENTR vectors were

digested with *Nsi*I, and their *Km*^r markers were replaced with a *Tmp*^r marker by ligation with the 0.9-kb *Pst*I *Tmp*^r marker fragment from pFTP2 to create pENTRTmp vectors. This allowed the recovery *E. coli* EPI300 clones on LB+*Km* plates with correct LR recombinants without recovering a *Km*^r pENTR background. In the second strategy, pENTR inserts were PCR amplified with primers M13F+M13R, which appends *att*L1 and *att*L2 sites to the cloned inserts. The linear PCR products were then gel purified and used in LR reactions with pFTCAPL-GW before transformation into *E. coli* EPI3000 and recovery on LB+*Km* plates. This PCR-based strategy is the one that we currently favor for the LR recombination of both left and right capture vectors, as it is both faster and less cumbersome. EPI300 pFTCAPL LR recombinant clones were induced to high copy numbers with 0.2% arabinose prior to plasmid purification and confirmed by *Bsr*GI and other restriction enzyme digests.

(ii) **Creation of double merodiploid donor strains.** Right and left capture vectors were transformed into the conjugation strain RHO3. Using RHO3 donors, capture vectors were conjugally transferred and homologously recombined into the *B. pseudomallei* strains at the right and left flanks by biparental mating on LB+DAP plates followed by recovery of merodiploid exconjugants with zeocin, gentamicin, or kanamycin selection as appropriate. Due to the occasional difficulty in recovering *Zeo*^r exconjugants with *AmrAB-OprA* efflux pump-proficient strains, the *Zeo*^r-conferring right capture vectors were always recombined into the *B. pseudomallei* strains first to simplify screening. Isolated exconjugant right capture vector merodiploid colonies were screened by PCR with the primer pair 536+537 for the presence of an *oriT*. Confirmed TCAPR *B. pseudomallei* merodiploids were then used as recipients in a second biparental mating to homologously recombine left capture vectors. Recovered *Km*^r exconjugant TCAPR TCAPL double merodiploids were then pooled and saved as *oriTn7* capture donors.

(iii) ***OriTn7* capture matings.** One ml each of LB+*Km*- or LB+*Km*+*Zeo*-grown cultures of double merodiploid *B. pseudomallei* donor strains, LB+*Rf*- or LB+*Rf*+*Tmp*-grown cultures of *E. coli* EPI300R1, and LB+*Km*- or LB+*Km*+*Gm*-grown cultures of DH5 α /pUCPRK2013 or DH5 α /pBBRK2013 helper strains was harvested by centrifugation and washed twice with 1 ml fresh LB medium prior to suspension in 100 to 200 μ l LB. Portions of 20 μ l each of donor, recipient, and helper strains were combined in a multiparental mating on a filter placed on an LB plate along with individual parental controls and incubated overnight at 37°C. *E. coli* exconjugants were recovered by selection on LB+*Rf*+*Tmp*+*Km*+*Zeo* plates. Candidate pFTarget-Tn7 clones were streaked for single-colony isolation on LB+*Zeo*+X-Gluc plates, and blue colonies were screened for *Km*^r, for *Gm*^s, and often for *Pmb*^s.

pFTarget-Tn7 vector confirmation. pFTarget-Tn7 vectors were confirmed by multiplex PCR, Eckhardt gel electrophoresis, and *I*-SceI restriction digestion. *E. coli* pFTarget-Tn7 candidate clones were inoculated into LB+*Km* or LB+*Zeo* medium with and without arabinose and grown overnight. Plasmid DNA was isolated and used as a template for multiplex PCR to confirm pFTarget-Tn7 insert identity. The multiplex primer set consisting of primers 2506 through 2519 was used to screen for the presence of the AMR, MBA, and 140 regions. Multiplex primers 2502 through 2505 were used to screen for the *B. pseudomallei* YLF and BTFC regions (24). Purified pFTarget-Tn7 plasmid DNA was analyzed by *I*-SceI restriction digestion to release the captured Tn7 target. The Eckhardt gel electrophoresis protocol as modified by the Griffiths lab (25) was used to resolve and visualize large pFTarget-Tn7 vectors. *Pseudomonas syringae* pv. phaseolicola 1448A 131.9-kb and 51.7 kb plasmids were used for size comparison (26). Briefly, overnight 30°C LB cultures of *P. syringae* pv. phaseolicola were subinoculated 2:7 in fresh LB and incubated for 4 h at 30°C with aeration to an optical density at 600 nm (OD₆₀₀) between 0.5 and 0.7. Overnight 37°C LB *Km* *E. coli* pFTarget-Tn7 cultures were subinoculated 1:10 in fresh LB+*Km* medium and incubated for 5 h at 37°C with aeration to an OD₆₀₀ between 0.5 and 0.7. Cell samples were chilled on ice, washed with 0.3% Sarkosyl, and lysed in the wells. The Eckhardt gel (1× SBE [10 mM NaOH, 1 mM EDTA, 29 mM boric acid,

pH 8.0], 0.9% agarose, 0.5% SDS) was run in 1× SBE buffer at 100 V for 2.5 h total with 30 min current-on periods and 15-min current-off rest periods to prevent melting/warping of the gel. The gel was stained with SYBR Safe DNA gel stain from Invitrogen Life Technologies according to the manufacturer's recommendations.

pFTarget-Tn7 transposition. To perform pFTarget-Tn7 transposition, pFTarget-Tn7 vectors were transferred into derivatives of the RHO3 conjugation strain, which can be metabolically counterselected by plating on LB medium lacking DAP. For pFTarget-Tn7 vectors with inserts of less than 15 kb, copy control-induced plasmid DNA was electroporated into RHO5, and transformants were recovered on LB+*Km*+DAP. To avoid deletion of insert sequences that routinely occurred when pFTarget-Tn7 vectors were retransformed with the 140-kb insert into RHO3, these plasmids were transferred into RHO3cm via conjugation. EPI300R1 pFTarget-Tn7 was used as the donor, DH5 α /pBBRK2013 as the conjugation helper, and RHO3cm/pBBRSac3 as the recipient. The pBBRSac3 vector acts as an exclusion plasmid via plasmid incompatibility to prevent RHO3cm exconjugants from receiving the pBBRK2013 conjugation helper plasmid. Exconjugants were recovered on LB+DAP+*Km*+*Zeo*+*Cm*+*Amp* plates and then streaked for single-colony isolation on LB+DAP+*Km*+*Zeo*+*Cm*+10% sucrose to cure pBBRSac3. Recovered clones were screened for *Km*^r, *Amp*^s, *Gm*^s, and DAP auxotrophy. Plasmid DNA was isolated from RHO3cm/pFTarget-Tn7 exconjugants and reconfirmed by multiplex PCR. RHO5 or RHO3cm/pFTarget-Tn7 donors and RHO3/pTNS3 transposition helpers were conjugated with *B. pseudomallei* recipients and recovered on LB+*Zeo* or LB+*Gm* plates as appropriate. pFTarget-Tn7 transposition candidates were screened for *Km*^r to eliminate single crossover events carrying the pFTarget-Tn7 backbone. Candidate Δ (*amrRAB-oprA*)-complemented strains were additionally screened for *Gm*^r. *B. pseudomallei* pFTarget-Tn7 integrant candidates were checked for Tn7 insertion at *attTn7*-1, -2, or -3 using primer 479 in combination with primer 1509, 1510, or 1511, respectively (23). *B. thailandensis* candidates were checked for Tn7 insertion at *glmS1* or *glmS2* with primer 479 and primer 618 or 619, respectively (27). The appropriate multiplex PCR described above was used to confirm the pFTarget-Tn7 insert. Siderophore production phenotypes of Tn7 transposition candidates and control strains were determined by a quantitative chrome azurol S assay as described previously (28) from overnight cultures grown at 37°C in CAA medium (29).

Maintenance of pFTarget-Tn7 as an RK2-based replicating plasmid. *B. thailandensis* strain BT36 was electroporated with pARATrfA, and transformants were recovered on LB+*Zeo* plates at 30°C. BT36 pARATrfA and BT36 plasmid-negative control recipients were combined with the RHO5/pFTarget2-Tn7-YLF donor in biparental matings on an LB plate augmented with DAP and ARA and incubated overnight at 30°C, and *Km*^r exconjugants were recovered. BT36 pARATrfA pFTarget2-Tn7-YLF maintenance was done at 30°C with ARA augmentation. To cure pARATrfA, BT36 pARATrfA pFTarget2-Tn7-YLF exconjugants were streaked for single-colony isolation on LB in the absence of selection at 42°C. Plasmid DNA was isolated from BT36/pARATrfA/pFTarget2-Tn7-YLF overnight cultures by boiling for PCR analysis and plasmid kit isolation for retransformation into *E. coli* EPI300R1.

RESULTS

To improve upon the *oriT*-directed capture system described by Chain et al., we constructed new left and right capture vectors and broad-host-range conjugation helpers, which facilitate the cloning, manipulation, and complementation of large specific genomic regions. The capture vectors pFTCAPL-GW and pUCTCAPR-GW were built to comply with *B. pseudomallei* select agent regulations, which restrict antibiotic marker use to *ble* (*Zeo*^r), *accCI* (*Gm*^r), and *nptII* (*Km*^r). However, they were also designed to allow easy swapping of the *Km*^r *uidA* and *Flp* recombinase target (FRT) *Zeo*^r markers in the pFTCAPL-GW and pUCTCAPR-GW vectors by *Eco*RI and *Xba*I digestion, respec-

FIG 1 OriTn7 capture system overview. (A) Separate mini-Tn7 capture suicide vectors based on pFTCAPL and pUUTCAPR are homologously recombined into the *B. pseudomallei* genome using cloned left and right regions that flank the target region. The GenBank accession numbers for pFTCAPL-GW, pUUTCAPR2-GW, and pUUTCAPR3-GW are JX573201, JX573199, and JX573200, respectively. (B) Transfer functions encoded by the broad-host-range conjugation helper mobilizes DNA downstream of the *oriT* into the recipient *E. coli* strain. (C) Transfer is halted at the second *oriT* site, and the plasmid is circularized in the recipient *E. coli* strain, generating a chimeric pFTarget-Tn7 vector carrying the target region. (D) Codelivery by conjugation of the pFTarget-Tn7 and the pTNS helper plasmid allows site-specific integration of the Tn7-functionalized target region into the *attTn7* site(s) of the chosen expression strain. *nptII*, kanamycin resistance gene; *aacCI*, gentamicin resistance gene; *ble*, zeocin resistance gene; *uidA*, GUS colorimetric marker; *glmS*, glucosamine-6-phosphate synthase gene; *oriT*, RP4 origin of transfer; I-SceI, I-SceI recognition site; L and R, left and right Tn7 ends; 1 and 2, Gateway *attB1* and *attB2* sites; *attTn7*, Tn7 insertion site; *FRT*, FLP recombinase target site; ccFOS, CopyControl RK2 inducible replication origin and mini-F replication origin; pUC, pUC replication origin; Left and Right, left and right target-flanking regions of homology.

tively. Rather than use the ColE1-based pRK2013 conjugation helper plasmid to establish non-*E. coli* strains as conjugation donors, we created two new broad-host-range conjugation helper plasmids, pUCPRK2013 and pBBRK2013, by replacing the ColE1 replicon of pRK2013 with either the pRO1600 replicon-containing vector pUCP24 or the *Bordetella* BBR replicon-containing vector pBBR1MCS-5. These vectors both used the *accCI* and *nptII* resistance markers, which are compliant with specific regulations regarding *B. pseudomallei*.

The steps and features of the oriTn7 system are summarized in Fig. 1. In brief, segments of DNA flanking the target region to the left and right are cloned into pFTCAPL-GW and pUUTCAPR-GW via Gateway LR recombination. The capture vectors are then homologously recombined with their matching genomic regions, creating a merodiploid strain in which the target region is flanked by *oriT* sites (Fig. 1A). By using either the pUCPRK2013 or

pBBRK2013 conjugation helper plasmid, the merodiploid strain is then used as a conjugation donor so that the region between the *oriT* sites will be conjugated into an *E. coli* recipient strain (Fig. 1B). This results in the creation of a chimeric vector in which the target region is carried as cargo in a mini-Tn7 transposon and maintained by a mini-F replicon (Fig. 1C). The target region may then be inserted in a single copy into the *attTn7* site via Tn7 site-specific transposition for complementation or heterologous expression (Fig. 1D).

OriTn7 capture system efficacy testing. To test the efficacy of the oriTn7 capture system, two *B. pseudomallei* genomic regions of difference that cover a range of target sizes were selected for cloning.

The first region is an ~140-kb region encompassing the genes encoding the aminoglycoside and macrolide antibiotic AmrAB-OprA efflux pump and the malleobactin siderophore synthesis

FIG 2 Use of *oriTn7* capture to clone and complement regions associated with a large *B. pseudomallei* natural deletion. (A) Map of the approximately 140-kb region absent from the *B. pseudomallei* clinical isolate 708a. The relative locations of the *amrAB-oprA* aminoglycoside efflux pump-encoding genes, the efflux pump regulatory gene *amrR* (*amr*), and the malleobactin siderophore synthesis and uptake gene cluster (*mba*) are shown. The extents of left and right capture flanking regions for various targets are marked in gray. Multiplex probe regions 1 to 7 are in white and numbered. The three captured target regions, with their sizes, are shown below the region map. (B) Eckhardt gel electrophoresis of large circular pFTarget-Tn7 vectors carrying 140, MBA, and AMR targets. Expected sizes for target plus vector are as follows: 140, 155.1 kb; MBA, 77.2 kb; AMR, 24.4 kb. *Pseudomonas syringae* pv. phaseolicola 1448a (Pph) plasmids were used for size comparison. (C and D) Multiplex PCR confirmation of 140 and MBA target capture plasmids from *B. pseudomallei* 1710b and pFTarget-Tn7 integration into Bp338, a 1710b 140-kb deletion strain. Multiplex probe amplicons are numbered as in panel A. HL, HiLo DNA ladder. The 500-bp fragment is marked with an asterisk. PCR products in panel C, lane Δ140, are nonspecific amplicons. (E) Multiplex PCR confirmation of AMR target capture plasmid from *B. pseudomallei* 1710b and pFTarget-Tn7 integration into Bp338 708a, a clinical isolate carrying a natural 140-kb deletion. The multiplex probe amplicons are numbered as in panel A. The upper PCR product is a nonspecific amplicon, as in panel C, lane Δ140, seen only when *B. pseudomallei* genomic DNA was used as the multiplex PCR template. (F) Siderophore production and gentamicin resistance (10 μg/ml) phenotypes of duplicate complemented strains. Siderophore production complementation was determined by quantitative chrome azurol S assays of culture supernatants from cells grown in iron-limited medium. Means and standard deviations from triplicate measurements are shown.

and uptake cluster, which is absent from the clinical isolate 708a. Absence of the ~140-kb region has a minimal effect on virulence, as strains lacking this region remain fully lethal in a murine melioidosis model (30). For analysis of the ~140-kb deletion re-

gion, three targets corresponding to different functional portions of the region were captured from *B. pseudomallei* 1710b (Fig. 2). Although DNA sequence analysis using next-generation sequencing would have been an option for assessing the integrity of the

large cloned DNA fragments, we opted for more traditional technologies, such as PCR and phenotypic analyses, to assess integrity and functionality of cloned genes and operons.

A set of seven multiplex PCR probes, one probe approximately every 20 kb along the 140-kb region length, was used to confirm the presence of the captured targets (Fig. 2A). Eckhardt gel electrophoresis was used to resolve the large circular pFTarget-Tn7 plasmids to confirm their sizes and to confirm that a single plasmid was present in the capture recipients (Fig. 2B). Multiplex PCR using plasmid DNA templates confirmed the presence of the appropriate targets (Fig. 2C to E). The 140 target is 142.8 kb in size and captures the entire region absent from 708a. All seven multiplex probe sites are present in this target. The MBA region is 64.9 kb in size and captures the *amrAB-oprA* efflux pump operon and its associated regulatory gene *amrR*, as well as the malleobactin siderophore synthesis and uptake gene clusters. Multiplex probes 1 through 4 are located within this region. The AMR target is 12.1 kb in size and captures only the *amrAB-oprA* efflux pump operon and its *amrR* regulatory gene. Only multiplex probe site 1 is carried on this target. For each target region, the right capture flank was varied while the left capture flank was maintained. As it is occasionally difficult to cleanly recover Zeo^r exconjugants in AmrAB-OprA efflux pump-expressing strains, the Zeo^r -conferring right capture vector was always recombined into the chromosome first so that the merodiploid could be confirmed by checking for the presence of the *oriT* site by PCR. For 10^8 recipient cells, the number of recovered exconjugants from capture matings generally decreased with the size of the target from 10^2 to 10^3 for the AMR target to 10^1 for the 140 target.

The capacity of the system for complementation was tested in Bp338, a *B. pseudomallei* 1710b strain with a laboratory-generated deletion that mimics the ~ 140 -kb natural deletion in *B. pseudomallei* 708a. We were able to successfully recover Tn7 integrants of all three pFTarget-Tn7 regions into Bp338 with frequencies ranging from 10^2 to 10^3 exconjugants per $\sim 10^8$ recipient cells. Km^s Tn7 integrants were identifiable from the background of Km^r single-crossover exconjugants at approximately 1:10 for the MBA and 140 target regions and 1:3 for the AMR target region. The integrants were checked for *attTn7* insertions by PCR, and target presence was confirmed by multiplex PCR (Fig. 2C to E). Similar attempts were made to recover Tn7 integrants in *B. pseudomallei* 708a, but Tn7 integrants were successfully recovered only with the AMR target region. For the MBA and 140 target regions, only Km^r capture flank region crossover exconjugants of *B. pseudomallei* 708a were recovered (Fig. 2A). Other than acknowledging that strain 1710b is easier to genetically manipulate than other *B. pseudomallei* strains, we do not understand the reason(s) for our inability to recover site-specific mini-Tn7 integrants carrying the MBA and 140 regions. pFTarget-Tn7 integrants were also phenotypically confirmed. Resistance to Gm was restored in Tn7 integrants with any of the three target regions, but siderophore production was restored only in Tn7 integrants carrying either the MBA or 140 target (Fig. 2F). These experiments confirmed the malleobactin siderophore phenotypes previously associated with the 140-kb region deletion either naturally present in strain 708a or genetically engineered in the strain 1710b background (31). More importantly, the newly developed large fragment capture technology allowed us to do what we could not experimentally achieve previously, that is, to successfully complement the naturally present or engineered large genetic lesions causing the ob-

served malleobactin siderophore deficiency and AmrAB-OprA efflux pump susceptibility phenotypes (31).

The second test region of 11 kb is the *Yersinia*-like fimbrial (YLF) gene cluster, which is one of a pair of *B. pseudomallei* gene clusters whose presence is geographically biased (24). The YLF and *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene clusters are found at the same chromosomal location in different strains and appear to be mutually exclusive in nature (24). The YLF cluster is more common in Thai *B. pseudomallei* isolates, while Australian *B. pseudomallei* strains more commonly carry the BTFC cluster (24). *B. thailandensis* strains carry the BTFC cluster and provided the cluster's namesake. Phenotypes associated with the YLF cluster are unknown, but the BTFC cluster includes the gene *motA2*, which has been shown to be essential for intracellular motility and intercellular spread by *B. thailandensis* in the absence of the BimA actin recruitment-based motility system (32). Both YLF- and BTFC-carrying strains cause human disease and are virulent in animal models. Thus, neither of these regions carries genes that would be expected to enhance virulence of a strain from which either the YLF or BTFC cluster is absent (24, 33).

As we had already created *B. thailandensis* and Australian *B. pseudomallei* strain derivatives with deletions of their native BTFC gene clusters, we sought to capture the 11.4-kb YLF gene cluster from the Thai *B. pseudomallei* isolate 1026b and use Tn7 integration to create sets of strains carrying various combinations of the two gene clusters. The YLF target insert was sufficiently small that the I-SceI digest fragments of pFTarget2-Tn7-YLF were resolved and confirmed by standard gel electrophoresis (Fig. 3A). pFTarget-Tn7 integration was conducted and confirmed by multiplex PCR in the Australian *B. pseudomallei* isolate MSHR305 and its ΔBTFC derivative as well as by PCR in *B. thailandensis* E264 and its ΔBTFC derivative (Fig. 3B and C) (32). Thus, we have generated sets of isogenic *B. pseudomallei* and *B. thailandensis* strains carrying neither gene cluster, their native BTFC cluster, their nonnative YLF cluster, or both gene clusters in combination. The ability to clone the YLF and BTFC gene clusters into defined *B. pseudomallei* genetic backgrounds will facilitate future characterization of the roles that the respective clusters play in this bacterium's biology.

Plasmid-based maintenance of pFTarget-Tn7 vectors. As an alternative to Tn7 integration, we sought to determine if the pFTarget-Tn7 vectors could be maintained as broad-host-range replicating plasmids by utilizing the RK2 replication origin on the ccFOS vector backbone. Plasmid replication from the RK2 origin requires expression of the TrfA protein, which can be provided *in trans*. To this end, we created pAR trfA , a temperature-sensitive *Burkholderia* P $_{\text{BAD}}$ -*trfA* expression vector, as a RK2 replication helper. We chose this strategy to aid in our analyses, as it would result in temperature-sensitive replication of pFTarget-Tn7 (Fig. 4A). For stable pFTarget-Tn7 plasmid maintenance, the use of a non-temperature-sensitive constitutive TrfA expression replication helper would be preferable. *B. thailandensis* BT36 was transformed with pAR trfA and used as a conjugation recipient with a RHO5/pFTarget2-Tn7-YLF donor. Exconjugants were recovered at 30°C on LB+Km+ARA plates to maintain the plasmids. Candidates were purified at 42°C to cure both plasmids. All 12 single colonies tested were sensitive to both Zeo and Km. Control conjugations with BT36 lacking pAR trfA resulted in the recovery of a small number of presumed crossover exconjugants whose Zeo and Km resistance could not be cured at 42°C. PCR confirmed

FIG 3 Creation of *Burkholderia* strains carrying geographically diverse gene clusters. (A) I-SceI digest of pFTarget-Tn7 vectors carrying the AMR and YLF targets. Expected sizes are as follows: AMR, 10.4 kb and 14 kb; YLF, 10.4 and 13.2 kb. HL and λ refer to HiLo and lambda monocut mix DNA ladders, respectively. (B and C) Multiplex PCR and PCR confirmation of YLF gene cluster capture and the creation of *B. pseudomallei* MSHR305 and *B. thailandensis* E264 strains carrying the YLF gene cluster. The YLF probe size was 350 bp; the BTFC probe size was 115 bp. Note that the BTFC probe will not amplify from the native *B. thailandensis* BTFC cluster (24). The 500-bp fragment is marked with an asterisk.

both the presence of the YLF region in BT36/pFTarget2-Tn7-YLF plasmid-replicating exconjugants and the loss of YLF in temperature-cured strains (Fig. 4B). Plasmid DNA purified from pFTarget2-Tn7-YLF-replicating BT36 was transformed into *E. coli* EPI300R1. Comparison between I-SceI digests of plasmid DNA from the original pFTarget2-Tn7-YLF and recovered *E. coli* clones indicated that pFTarget-Tn7-YLF was recovered intact from BT36 (Fig. 4C).

Based on our initial success, we sought to determine if pARAtfA would allow large-insert pFTarget-Tn7 vectors to be maintained as replicating plasmids in *B. pseudomallei* 708a or if pARAtfA might enhance Tn7 integration of large target inserts into 708a by allowing even transient pFTarget-Tn7 replication. Unfortunately, while pARAtfA enhanced the recovery of Km^r 708a flank region crossover exconjugants, it did not allow the temperature-sensitive replication of large target MBA or 140-kb insert plasmids or the recovery of large-insert Tn7 integrants.

DISCUSSION

The oriTn7 capture system offers some key advantages for the specific cloning and downstream manipulation of genomic frag-

FIG 4 Maintenance of pFTarget-Tn7 as a plasmid in *B. thailandensis*. (A) Expression of TrfA from the *Burkholderia* temperature-sensitive P_{BAD} expression plasmid allows replication from the CopyControl RK2 origin on the ccFOS backbone of pFTarget-Tn7. Temperature-based curing of one pARAtfA results in curing of the pFTarget-Tn7 vector. (B) PCR confirmation of YLF cluster presence in pFTarget-Tn7 plasmid-maintaining *B. thailandensis* BT36 grown at 30°C. (C) I-SceI digests of pFTarget2-Tn7-YLF alongside transformant clones recovered from BT36/pARAtfA pFTarget2-Tn7-YLF purified plasmids. HL and λ refer to HiLo and lambda monocut mix DNA ladders, respectively.

ments over other large-fragment cloning systems. The oriTn7 capture system can easily accommodate fragment sizes from 10 to over 100 kb and is mediated by reliable bacterial conjugation. All manipulation of large DNA is conducted *in vivo*, which eliminates

the issue of DNA fragmentation. Finally, the captured target region is functionalized with Gateway *attB* sites and I-SceI recognition sites for transfer into other vectors and is also functionalized as a mini-Tn7 for single-copy chromosomal integration or maintenance as a multicopy plasmid by using the RK2 origin of replication.

Details of specific methodology considerations for the steps of the oriTn7 capture system are discussed below. First, to create target region-specific capture vectors, left and right capture regions flanking the desired target region are PCR amplified and TOPO cloned into directional pENTR vectors. Left and right flanking region primers are designed to amplify flanking regions of sufficient size to facilitate single homologous crossover events. In addition, flanking regions corresponding to central fragments of genes may be undesirable, as they will create corresponding gene interruptions after homologous recombination. The forward primers for each flanking region have the 5' CACC directional TOPO sequence appended such that the correct flanking region orientations are maintained with respect to the targets genomic orientation throughout the procedure. The left and right flanking regions carried in the pENTR vectors are then LR recombined with the left (pFTCAPL-GW) and right (pUCTCAPR2-GW or pUCTCAPR3-GW) capture vectors (Fig. 1A).

The left and right capture vectors are sequentially recombined into the target genome via homologous recombination, resulting in a double merodiploid donor strain with two directly repeated *oriT* sites flanking the target region (Fig. 1B). The mini-F and pUC plasmid replicons in the left and right capture vectors are incapable of replication in *Burkholderia* spp. and many other bacteria. This allows antibiotic selection to identify RecA-mediated homologous recombination events. Exconjugant double merodiploid recombinants with both capture vectors recombined into the chromosome are pooled for use in subsequent capture matings.

Using one of the newly constructed broad-host-range conjugation helpers, pUCPRK2013 or pBBRK2013, conjugation is initiated from one integrated *oriT* site of the double merodiploid donor strain, and a single strand is transferred into the *E. coli* recipient strain. Conjugation continues until the second integrated *oriT* is reached. This halts the conjugation process, resulting in the creation of a chimeric circularized, Km^r Zeo^r, and Gm^s pFTarget-Tn7 vector carrying the desired target region (Fig. 1C). Although both broad-host-range conjugation helpers has proved effective for capture matings, in general, the use of pBBRK2013 results in 2-fold-greater numbers of recovered exconjugants from *B. pseudomallei*. Conjugation initiated from the second *oriT* is nonproductive, as it would require conjugation of the entire chromosome.

Recovered pFTarget-Tn7 candidate exconjugants are purified and screened for Gm^s. This screen checks for donor breakthrough, recovery of both the evicted capture vectors in combination, and the recovery of the pFTarget-Tn7 vector in combination with a conjugation helper plasmid. This last option was the most common cause of Gm^r exconjugants. In general, Gm^s exconjugant clones appear to be recovered at a higher percentage as the size of the target insert increases. For example, for the 140-kb fragment, ~75% of the exconjugants were Gm^s, whereas for the 12.1-kb AMR fragment, ~25% were Gm^s. However, if no Gm^s clones are recovered from a capture mating, small insert pFTarget-Tn7 vectors (15 kb or less) can be isolated and retransformed by electroporation. Additionally, for large-insert pFTarget-Tn7 vectors,

conjugation from an EPI300R1/pFTarget-Tn7 pBBRK2013 donor into a RHO3cm/pBBRsac3 BBR exclusion recipient can be used to recover clones lacking the pBBRK2013 helper plasmid.

In the final step of constructing a Tn7 integrant expression or complementation strain, the pFTarget-Tn7 vector is mobilized by conjugation into the desired recipient strain in combination with a Tn7 transposase-encoding helper plasmid. The expression of the site-specific Tn7 transposition pathway proteins is necessary to integrate the target Tn7 at *glmS*-associated *attTn7* sites (Fig. 1D) (27). Putative Tn7 integrants are screened for single-crossover events by checking for Km^s. For inserts under 15 kb, Tn7 integrants are easily recovered, presumably because the rate of Tn7 transposition for inserts of this size is higher than the rate of RecA-mediated recombination with the genome. To successfully recover *attTn7*-located integrants for large inserts, RecA-mediated crossover between the target insert and the genome must be restricted. This can be accomplished by limiting the homology between the target insert and genome by using a strain carrying a matching deletion of the target insert, by using a strain with limited genome homology to the target, or by integrating into a *recA*-deficient strain.

Although the left and right capture vectors by necessity share homology at their *oriT* sites, efforts were made to keep intervector homology to a minimum to prevent RecA-mediated homologous recombination between the two capture vectors during the creation of double merodiploid donor strains. Cloning larger target-flanking regions will help bias against inter-*oriT* homologous recombination, and it would also typically be favorable to homologously recombine the capture vector carrying the smaller of the two flanking regions first rather than second. However, a capture mating from a donor with inter-*oriT* recombined capture vectors would only result in the recreation of the original misrecombined capture vector after conjugation into the *E. coli* recipient. As the original capture vectors do not individually carry the required combination of antibiotic markers to be recovered, pooling the double merodiploid exconjugants is a desirable strategy for this step of the system.

The final captured target regions carried in pFTarget-Tn7 vectors are usefully functionalized in various ways. The pFTarget-Tn7 vectors carry both a mini-F-based replicon, which can easily accommodate inserts of over 100 kb, and an RK2 *oriV*, which allows broad-host-range replication of pFTarget-Tn7 vectors if TrfA is provided in *trans* or induction to high copy number in CopyControl strains that express a mutant, copy-up variant of the TrfA protein (34). The single *oriT* site on the pFTarget-Tn7 vectors both is intrinsic to the system and facilitates conjugation-based mobilization of large pFTarget-Tn7 vectors between strains. The incorporation of Gateway cassettes into the left and right capture vectors in combination with directional pENTR/D-TOPO vectors simplifies work flow and primer design but also results in the captured target region being flanked with Gateway *attB* sites. The *attB* sites can be used to recombine the target region into *attP* carrying Gateway pDONR vectors, assuming that the particular pDONR vector is capable of supporting replication of the target insert. The target Tn7 is flanked by 18-bp, rare cutting I-SceI endonuclease recognition sites, which both aid in restriction-based screening of candidates and allow I-SceI-based restriction subcloning of targets inserts. Lastly, the pFTarget-Tn7 element carries a FLP recombinase-excisable FRT-Zeo^r-FRT cassette. This allows the target insert to be site-specifically transposed in a

single copy into the chromosomes of most Gram-negative bacteria, followed by Flp-mediated excision of the *Zeo^r* selection marker. Some bacteria, for instance, *B. thailandensis* and *B. pseudomallei*, contain more than one Tn7 insertion site, but simultaneous insertion into multiple sites occurs less frequently than single-site insertion, and single and multiple insertions can easily be differentiated by PCR.

In addition, if both capture flanking regions have homology to the borders of a matching chromosomal deletion, the pFTarget-Tn7 vector may also be used as an allelic exchange vector for deletion restoration. The I-SceI recognition sites in the vector backbone can serve as counterselection markers in combination with an I-SceI endonuclease expression plasmid (35). Colorimetric screening for the *uidA* gene would aid the identification of double-crossover events resolving the merodiploid state. Unfortunately, this strategy cannot be used in *B. pseudomallei* strains expressing AmrAB-OprA, as all three currently permissible antibiotic resistance markers—*Gm^r*, *Km^r*, and *Zeo^r*—are already in use, leaving none to select for introduction of an I-SceI expression plasmid. However, one could envision the use of other permissible non-antibiotic resistance markers, such as glyphosate resistance, for maintenance of the I-SceI expression vector (36).

Currently, the *oriTn7* capture system is limited to use in genetically tractable Gram-negative bacteria that can be established, at least transiently, as conjugation donors. The F replicon and the pUC replicon must also be nonreplicative in the target organism. Even with these limitations, the genera *Burkholderia*, *Pseudomonas*, *Ralstonia*, and *Sinorhizobium* should all be compatible, and likely many other genera as well.

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