APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2008, p. 1064–1075 Vol. 74, No. 4
0099-2240/08/$08.00+0 doi:10.1128/AEM.02430-07

Genetic Tools for Select-Agent-Compliant Manipulation of *Burkholderia pseudomallei*▽

Kyoung-Hee Choi,[1] Takehiko Mima,[1] Yveth Casart,[2]† Drew Rholl,[1] Ayush Kumar,[1]‡ Ifor R. Beacham,[2] and Herbert P. Schweizer[1]*

Department of Microbiology, Immunology and Pathology, Rocky Mountain Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research, Colorado State University, Fort Collins, Colorado 80523-1682,[1] and School of Medical Science and Institute for Glycomics, Griffith University—Gold Coast Campus, Gold Coast, Queensland 9726, Australia[2]

Received 28 October 2007/Accepted 13 December 2007

Because of *Burkholderia pseudomallei*'s classification as a select agent in the United States, genetic manipulation of this bacterium is strictly regulated. Only a few antibiotic selection markers, including gentamicin, kanamycin, and zeocin, are currently approved for use with this bacterium, but wild-type strains are highly resistant to these antibiotics. To facilitate routine genetic manipulations of wild-type strains, several new tools were developed. A temperature-sensitive pRO1600 broad-host-range replicon was isolated and used to construct curable plasmids where the Flp and Cre recombinase genes are expressed from the rhamnose-regulated *Escherichia coli* P_{BAD} promoter and kanamycin (*nptI*) and zeocin (*ble*) selection markers from the constitutive *Burkholderia thailandensis* ribosomal P_{S12} or synthetic bacterial P_{EM7} promoter. Flp and Cre site-specific recombination systems allow in vivo excision and recycling of *nptII* and *ble* selection markers contained on *FRT* or *loxP* cassettes. Finally, expression of Tn*7* site-specific transposase from the constitutive P1 integron promoter allowed development of an efficient site-specific chromosomal integration system for *B. pseudomallei*. In conjunction with a natural transformation method, the utility of these new tools was demonstrated by isolating an unmarked Δ(*amrRAB-oprA*) efflux pump mutant. Exploiting natural transformation, chromosomal DNA fragments carrying this mutation marked with zeocin resistance were transferred between the genomes of two different *B. pseudomallei* strains. Lastly, the deletion mutation was complemented by a chromosomally integrated mini-Tn*7* element carrying the *amrAB-oprA* operon. The new tools allow routine select-agent-compliant genetic manipulations of *B. pseudomallei* and other *Burkholderia* species.

Burkholderia pseudomallei is the etiologic agent of melioidosis (12, 61). Melioidosis is endemic in Southeast Asia and Northern Australia, but more recent studies indicate that the disease is probably pandemic between 20°N and 20°S (12, 46). Cases elsewhere in the world are rare and almost always attributable to travel to regions of endemicity (3, 31). The genome sequences of several *B. pseudomallei* strains have been determined (40) (several GenBank entries), and efforts are being initiated to take advantage of this information to learn more about the biology and pathogenesis of these bacteria. Prior to its designation in the United States as a category B select agent, there had been steady progress toward the development and use of genetic methods for manipulating *B. pseudomallei* (22, 23, 39, 53). In the United States, however, these efforts are now severely hampered because of strict select-agent guidelines which restrict the use of antibiotic resistance selection markers to those that do not conflict with application of the respective antibiotics in human and veterinary medicine as well as agriculture. At the time of writing of this paper, only gentamicin (Gm), kanamycin (Km), and zeocin (Zeo) were approved for use in genetic manipulation of *B. pseudomallei*. All three, unfortunately, are of limited usefulness because wild-type strains are intrinsically resistant to these antibiotics due to efflux by the constitutively expressed AmrAB-OprA pump (39; A. Kumar, K.-H. Choi, and H. P. Schweizer, unpublished observations). As a consequence of limited selection marker availability, routine genetic manipulations of these pathogens, including mutant construction, complementation analyses, transposon mutagenesis, etc., are cumbersome and not yet a matter of routine.

Site-specific excision of selection markers allows recycling of the few precious markers available for many bacteria. Useful methods previously employed with bacteria for in vivo site-specific excision of unwanted selection markers from the chromosome involve the *Saccharomyces cerevisiae* Flp-*FRT* system (13, 47), the Cre-*lox* system of the P1 phage/plasmid (1, 55), the ParA-*res* system of RP4 (32), and the TnpR-*res* system of Tn*1000* (or γδ transposon) (7). Of these, the Flp-*FRT* and Cre-*lox* systems are perhaps the most versatile, functioning in bacteria, yeasts, plants, insects, and mammalian cells (27, 35, 42, 49, 54, 57). Site-specific recombination systems minimally require two components: (i) a target genetic element flanked by recombination sites and (ii) a genetic element encoding the recombinase. For bacteria, the recombinase source is typically encoded by a curable plasmid and plasmid curing is generally achieved by use of a counterselection marker, e.g., *Bacillus*

* Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682. Phone: (970) 491-3536. Fax: (970) 491-1815. E-mail: Herbert.Schweizer@colostate.edu.

† Present address: Departamento de Biología Estructural, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela.

‡ Present address: Faculty of Health Sciences, 2000 Simcoe St. N, University of Ontario Institute of Technology, Oshawa, Ontario L1H 7K4, Canada.

▽ Published ahead of print on 21 December 2007.

subtilis sacB (reviewed in reference 52) or a temperature-sensitive (TS) replicon (37, 44). To date, neither one of these is applicable for *B. pseudomallei*, because most *Burkholderia* spp. contain endogenous *sacB* genes and no TS plasmid replicon is available.

The recent availability of the genomic sequences of several *B. pseudomallei* strains (29) (several GenBank entries) provides new opportunities for understanding the biology and pathogenesis of these opportunistic yet highly fatal pathogens. To take full advantage of the wealth of information contained in these genomes, however, new genetic tools that are in compliance with select-agent guidelines need to be developed. Specifically needed are recyclable selection markers and curable plasmids for mutant construction, transfer of mutations between chromosomes, regulatable and constitutive promoters, and site-specific chromosome integration systems. The genetic tools and accompanying methods described in this paper address all of these needs. In this study, we report the isolation of a TS replicon and its use for establishment of in vivo Flp-*FRT* and Cre-*loxP* site-specific recombination systems for *B. pseudomallei* and other *Burkholderia* spp. Our results demonstrate that together with corresponding Gm, Km, and Zeo marker cassettes and exploitation of natural transformation, these new tools allow routine and select-agent-compliant genetic manipulation of *B. pseudomallei* with the few antibiotic selection markers currently approved for use with these bacteria. Examples are provided for the usefulness of these tools for isolation of an unmarked efflux pump mutant, transfer of this mutation between chromosomes of different strains, and establishment of a site-specific chromosomal integration system.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Several *Burkholderia* strains were used in this study. Before the use of *B. pseudomallei*, genetic constructs were routinely tested with *Burkholderia thailandensis* wild-type strain E264 (4). *B. thailandensis* is traditionally regarded as a naturally attenuated relative of *B. pseudomallei* (63) which can be handled at biosafety level 2 and is exempt from select-agent guidelines. The *B. pseudomallei* strains used were 1026b, its Δ(*amrAB-oprA*) derivative DD503 (39), and 1710b (Table 1). The *Escherichia coli* strains used for routine cloning experiments were DH5α (34), HPS1 (51), and strains provided with specific cloning kits. All bacteria were routinely grown at 37°C. Strains containing TS plasmid derivatives were grown at 30°C (permissive temperature) or 42°C (nonpermissive temperature). LB broth Miller and LB agar Miller from EMD Chemicals (Gibbstown, NJ) were used as the rich media for all bacterial strains, except for propagation and selection of Zeo-resistant strains, for which low-salt (5 g liter^{-1} NaCl) Lennox LB broth (LSLB) and agar (MO BIO Laboratories, Carlsbad, CA) were used. For preparation of the competent *B. pseudomallei* cells used for transformation with linear DNA fragments, bacteria were grown in DM medium, which consists of 0.25× M63 (38) supplemented with 0.2% glucose, 0.4% glycerol, 1 mM $MgSO_4$, 1 μg ml^{-1} thiamine-HCl, and 40 μg ml^{-1} each of leucine, isoleucine, valine, tryptophan, glutamic acid, and glutamine, as described previously (M. Thongdee, L. A. Gallagher, T. Dharakul, S. Songsivilai, and C. Manoil, presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand). Unless otherwise noted, antibiotics were added at the following concentrations: 100 μg ml^{-1} ampicillin (Ap), 35 μg ml^{-1} Km, 10 μg ml^{-1} Gm, 100 μg ml^{-1} trimethoprim (Tp), and 25 μg ml^{-1} Zeo for *E. coli*; 700 to 1,000 μg ml^{-1} Km and 2,000 μg ml^{-1} Zeo for wild-type *B. pseudomallei* and 100 μg ml^{-1} Km or 50 μg ml^{-1} Gm for Δ(*amrRAB-oprA*) mutants; and 200 μg ml^{-1} Zeo, 100 μg ml^{-1} Tp, and 100 μg ml^{-1} Km for *B. thailandensis*. Antibiotics were purchased from Sigma, St. Louis, MO (Ap, Km, and Tp), EMD Biosciences, San Diego, CA (Gm), and Invitrogen, Carlsbad, CA (Zeo).

Antimicrobial-susceptibility testing. MICs were determined by the twofold broth microdilution technique, following Clinical and Laboratory Standards Institute guidelines (21).

DNA methods and transformation procedures. Routine procedures were employed for manipulation of DNA (48). Plasmid DNAs were isolated from *E. coli* and *Burkholderia* spp. by using a QIAprep mini-spin kit (Qiagen). Bacterial chromosomal DNA fragments (20 to 30 kb) were isolated using a QIAamp DNA mini kit, and the DNA was suspended in 200 μl of buffer AE (10 mM Tris-HCl, 0.5 M EDTA, pH 9). Plasmid DNA fragments were purified from agarose gels by utilizing a Qiagen QIAquick gel extraction kit. *E. coli* strains were transformed using chemically competent cells (48). Replicative plasmids were transformed into *B. thailandensis* and *B. pseudomallei* by using the rapid electroporation procedure previously described for *Pseudomonas aeruginosa* (16). Custom oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). DNA maps were constructed using Gene Construction Kit 2.5 (Textco, West Lebanon, NH) and then exported to Microsoft PowerPoint for final annotation.

Isolation of a TS pRO1600 replicon. The pRO1600 wild-type replicon was mutagenized by error-prone PCR using pUCP28T containing the ori_{1600}-*rep* sequences on a 1.2-kb fragment as the template and primers rep-UP (5′-TTCG TGAATTCGCCGCTGGT) and rep-DN (5′-GGCCTCTAGGCCAGATCCA). A 100-μl PCR mixture contained 100 ng of pUCP28T template DNA, 1× *Taq* buffer, 0.7 mM $MgCl_2$, 0.3 mM $MnCl_2$, 20 μM of dATP and dGTP, 0.1 mM of dCTP and dTTP, 0.51 μM of rep-UP and rep-DN, 5% dimethyl sulfoxide, and 5 units of *Taq* DNA polymerase (New England Biolabs). This reaction mixture was divided into 10-μl aliquots, which were then subjected to the following cycle conditions: one cycle of 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min 25 s; and a final extension at 72°C for 10 min. The resulting PCR products were ligated into the pCR2.1 TA cloning vector (Invitrogen), and the ligation mixes were transformed into *E. coli* DH5α cells. Transformants were selected on LB plates containing 100 μg ml^{-1} Ap. Transformants were pooled, and plasmid DNA was extracted. The pooled plasmid DNAs were digested with EcoRI and blunt ended with T4 DNA polymerase. The 1.2-kb ori_{1600}-*rep* fragment was ligated to ScaI-digested pFTP1 DNA and the transformation mixture transformed into *E. coli* HPS1. Transformants were selected at 37°C on LB plates containing 100 μg ml^{-1} Tp. For screening of plasmids containing a TS replicon, the plasmid pool obtained from these transformants was transformed into *B. thailandensis* E264, followed by selection of Tp-resistant (Tp^r) transformants at 30°C. Individual transformants were patched onto replicate Tp-containing plates, which were then incubated at 30°C and 42°C. Plasmid DNA was isolated from several colonies which grew at 30°C but not at 42°C and was used to retransform E264, followed by selection of Tp^r transformants and screening of TS phenotypes as described above. Five isolates were retained, and the entire sequence of the ori_{1600}-*rep* insert contained on the respective plasmids was determined using primers rep-UP and rep-DN, but only one (pPS2158) was retained for further studies.

Determination of plasmid copy number. The wild-type ori_{1600}-*rep* and ori_{1600}-*rep*(Ts_{Bt}) regions were amplified from pUCP28T and pPS2158, respectively, using primers rep-UP (5′-TTCGTGAATTCGCCGCTGGT) and rep-DN (5′-G GCCTCTAGGCCAGATCCA). The resulting PCR fragments were cloned into TA cloning vector pCR2.1 (Invitrogen), generating pPS2194 and pPS2163. These two plasmids were electroporated into *B. thailandensis* E264, and transformants were selected on LB medium containing 100 μg ml^{-1} Km. Total DNA was isolated from transformants by using the QIAamp DNA mini kit (Qiagen). Plasmid copy number was determined as described by Lee et al. (33) with minor modifications. With this method, the separate detections of a plasmid and host chromosomal DNA were achieved using two separate primer sets, specific for the plasmid β-lactamase gene (*bla*) and for the chromosomal, single-copy aspartate-β-semialdehyde dehydrogenase gene (*asd*). To this end, the *B. thailandensis asd* gene was first cloned into the *bla*-containing pCR2.1 TA cloning vector to form pPS2191 and the copy number of this plasmid was calculated from the plasmid concentration by using the following equation: DNA copy number = (6.02×10^{23} [no. of copies/mol] × plasmid DNA amount [g])/(plasmid DNA length [bp] × 660 [g/mol/bp]). Tenfold serial dilutions of this plasmid and real-time quantitative reverse transcription-PCR were then employed for creating the standard curves for the *asd* and *bla* genes, where threshold cycle values were plotted against log copy number. Primers were designed using Primer 3 software and were as follows: for *bla*, bla-RT-UP (5′-ATAAATCTGGAGCCGGTGAG) and bla-RT-DN (5′-CTACGATACGGGAGGGCTTA), yielding an 81-bp product; and for *asd*, asd-RT-UP (5′-ACACGTCGTTCGTGTAGTCG) and asd-RT-DN (5′-AAAACGAGACCACGCTCAAG), yielding a 99-bp product. Real-time quantitative reverse transcription-PCR amplifications of the total DNA samples from two separate *B. thailandensis* E264 cultures harboring either pPS2194 or pPS2163 were performed simultaneously. The absolute copy num-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
B. thailandensis		
E264	Wild-type strain	4
B. pseudomallei		
1026b	Wild-type strain; clinical isolate	22
DD503	Str^r; Δ(*amrRAB-oprA*)	39
1710b	Wild-type strain; clinical isolate	Ubon collection
Bp44	Zeo^r; 1026b with Δ(*amrRAB-oprA*)::*FRT-ble-FRT*	This study
Bp50	1026b with Δ(*amrRAB-oprA*)::*FRT*	This study
Bp72	Bp50 with mini-T7-LAC-$amrA^+B^+$-$oprA^+$ (unmarked)	This study
Bp73	Bp50 with mini-T7-LAC (unmarked)	This study
Bp74	Zeo^r; 1710b with Δ(*amrRAB-oprA*)::*FRT-ble-FRT*	This study
Plasmids		
pCre1	Ap^r; source of *cre* gene	C. Manoil
pET24d-*FLPe*	Km^r; source of *FLPe* gene	11
pFGM1[b]	Ap^r Gm^r; source of gentamicin *FRT-aacC1-FRT* cassette	15
pFLP2	Ap^r; source of Flp recombinase	28
pFRT2	Ap^r; *FRT* cassette vector	15
pFRT3	Ap^r; pFRT2 with NdeI site deleted	Laboratory collection
pFTP1[b]	Ap^r Tp^r; source of trimethoprim *FRT-dhfRII-FRT* cassette	15
pGEM-T Easy	Ap^r; TA cloning vector	Promega
pRK2013	Km^r; helper plasmid for mobilization of non-self-transmissible plasmids	25
pTNS2[b]	Ap^r; plasmid expressing *tnsABCD* from P_{lac}	15
pTNS3[b]	Ap^r; plasmid expressing *tnsABCD* from *P1* and P_{lac}	This study
pUC18T-mini-Tn*7*T[b]	Ap^r; mobilizable mini-Tn*7* base vector	15
pUC18T-mini-Tn*7*T-LAC[b]	Ap^r Gm^r; mini-Tn*7* vector with $lacI^q$-P_{tac} expression cassette	15
pUCP28T[b]	Tp^r; mobilizable broad-host-range cloning vector	53
pPS1418	Ap^r Km^r; pCR2.1 with *nptI* gene from pUC4K (GenBank accession no. X06404) with HindIII, NruI, SmaI, and XhoI sites eliminated by site-directed mutagenesis	This study
pPS1734	Ap^r Km^r Zeo^r; pCR2.1 with P_{EM7}-*ble* PCR fragment from pDONR (Invitrogen Gateway vector)	This study
pPS2036	Ap^r; pGEM-T Easy with *rhaS-rhaR-*P_{rhaBAD} PCR fragment	This study
pPS2142	Ap^r Gm^r; pUC18T-miniTn*7*T-LAC with $amrA^+B^+$-$oprA^+$; *amrAB-oprA* expression under P_{tac} control	This study
pPS2158	Ap^r Tp^r; pFTP1 with 1.2-kb ori_{1600}-*rep*(Ts_{Bt}) fragment	This study
pPS2163	Ap^r Km^r; pCR2.1 (Invitrogen TA cloning vector) with 1.2-kb ori_{1600}-*rep*(Ts_{Bt}) fragment from pPS2158	This study
pPS2165	Ap^r Km^r Zeo^r; pPS2163 with *ble* gene from pPS1734	This study
pPS2167	Ap^r Km^r; pPS1418 with ori_{1600}-*rep*(Ts_{Bt}) from pPS2158	This study
pPS2191	Ap^r Km^r; pCR2.1 with *B. thailandensis asd* gene PCR fragment	This study
pPS2194	Ap^r Km^r; pCR2.1 with 1.2-kb ori_{1600}-*rep* (wild-type) fragment	This study
pPS2201	Cm^r Tp^r; pACD4K-C (Sigma-Aldrich) with *FRT-dhfRII-FRT* inserted from pFTP1 inserted between *loxP* sites	This study
pPS2205	Ap^r; pGEM-T Easy with SOE PCR fragment combining *rhaS-rhaR-*P_{rhaBAD} from pPS2036 and *cre* from pCre1	This study
pPS2208	Ap^r; pCR2.1 with SOE PCR fragment combining *rhaS-rhaR-*P_{rhaBAD} from pPS2036 and *FLPe* from pET24d-FLPe	This study
pPS2225	Ap^r Km^r; pCR2.1 with 0.3-kb P1 promoter PCR fragment from p34ETp1 (23)	This study
pPS2230	Ap^r Tp^r; pUC18T-mini-Tn*7*T with *loxP-FRT-dhfRII-FRT-loxP* fragment from pPS2201	This study
pPS2254	Ap^r Zeo^r; pGEM-T Easy with Δ(*amrRAB-oprA*)::*FRT-*P_{EM7}-*ble-FRT* SOE fragment	This study
pPS2272	pGEM-T Easy with *nptII* gene from pCR2.1	This study
pFLPe2[b]	Ap^r Zeo^r; contains *rhaS-rhaR-*P_{rhaBAD}-*FLPe* from pPS2208, *ble-*ori_{1600}-*rep*(Ts_{Bt}) from pPS2165 and *oriT-ori* from pFLP2	This study
pFLPe3	Ap^r Km^r; contains *rhaS-rhaR-*P_{rhaBAD}-*FLPe* from pPS2208, *nptI-*ori_{1600}-*rep*(Ts_{Bt}) from pPS2167 and *oriT-ori* from pFLP2	This study
pFLPe4[b]	Ap^r Km^r; pFLPe3 with P_{S12} promoter inserted in front of *nptI*	This study
pFLPe5	Ap^r Km^r; pFLPe3 with *nptII* from pPS2272	This study
pCRE3[b]	Ap^r Zeo^r; contains *rhaS-rhaR-*P_{rhaBAD}-*cre* from pPS2205, *ble-*ori_{1600}-*rep*(Ts_{Bt}) from pPS2165, and *oriT-ori* from pFLP2	This study
pCRE5[b]	Ap^r Km^r; contains *rhaS-rhaR-*P_{rhaBAD}-*cre* from pPS2205 and P_{S12}-*nptI-*ori_{1600}-*rep*(Ts_{Bt})-*oriT-ori* from pFLPe4	This study
pFKM2[b]	Ap^r Km^r; pFRT3 with *nptII* gene from pPS2272	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant properties[a]	Reference or source
pFZE1[b]	Ap^r Zeo^r; pFRT2 with P_{EM7}-*ble* fragment from pPS1734	This study
pLOX1[b]	Ap^r; *loxP* cassette vector with duplicated *loxP* sites from pACD4K-C and backbone of pFRT3	This study
pLKM1[b]	Ap^r Km^r; pLOX1 with *nptII* fragment from pPS2272	This study
pLZE1[b]	Ap^r Zeo^r; pLOX1 with P_{EM7}-*ble* fragment from pPS1734	This study
pUC18T-mini-Tn*7*T-Km-*lox*[b]	Ap^r Km^r; pUC18T-mini-Tn*7*T with *loxP-nptII-loxP* fragment from pLKM1	This study
pUC18T-mini-Tn*7*T-Zeo-*lox*[b]	Ap^r Zeo^r; pUC18T-mini-Tn*7*T with *loxP*- P_{EM7}-*ble-loxP* fragment from pLZE1	This study
pUC18T-mini-Tn*7*T-Km-*FRT*[b]	Ap^r Km^r; pUC18T-mini-Tn*7*T with *FRT-nptII-FRT* fragment from pFKM2	This study
pUC18T-mini-Tn*7*T-Zeo-*FRT*[b]	Ap^r Zeo^r; pUC18T-mini-Tn*7*T with *FRT*- P_{EM7}-*ble* -*FRT* from pFZE1	This study

[a] P_{lac}, P_{tac}, P_{EM7}, P_{rhaBAD}, and P_{S12} are *E. coli lac* operon, *E. coli lac* and *trp* operon hybrid, synthetic bacterial EM7, *E. coli rhaBAD* operon, and *B. thailandensis* ribosomal S12 protein gene promoters, respectively.

[b] Plasmids whose sequences were determined and deposited in GenBank for this study (for accession numbers, see Materials and Methods).

bers of *bla* and *asd* genes in the *B. thailandensis* total DNA samples were determined from the respective standard curves, using the threshold cycle values. The plasmid copy numbers of pPS2194 or pPS2163 were calculated by dividing the absolute copy number of *bla* by the copy number of *asd*. Experiments were repeated three times for calculation of copy number.

Promoter cloning. The *B. thailandensis* ribosomal protein S12 gene promoter (P_{S12}) or the P1 integron promoter (*P1*) were used for constitutive gene expression in *Burkholderia* spp. The two complementary synthetic oligonucleotides S12-UP (5′-GAGCTGTTGACTCGCTTGGGATTTTCGGAATATCATGCCGGGTGggcc) and S12-DN (5′-CACCCGGCATGATATTCCGAAAATCCCAAGCGAGTCAACAGCTCCtgca), specifying the P_{S12}-containing region (predicted −35 and −10 boxes are underlined) from *B. thailandensis* E264 (62), were annealed. The resulting double-stranded oligonucleotide contains ApaI and PstI restriction enzyme overhangs (indicated by lowercase letters in the primer sequences) flanking the promoter sequences for direct subcloning into target plasmids by use of the same sites. The P1 integron promoter was PCR amplified from p34ETp1 (23) on a 383-bp PCR fragment by using primers P1-UP (5′-GAATTCACGAACCCAGTTGAC) and P1-Sma1-DN (5′-TACTTTGACCCGGGTCGAATC) (the EcoRI and SmaI sites are underlined), and the fragment was cloned into pCR2.1 to form pPS2225. The *E. coli rhaBAD* operon promoter (P_{rhaBAD}) was used for regulated expression of genes in *Burkholderia* spp. (8). The region containing P_{rhaBAD} and its upstream regulatory genes *rhaS* and *rhaR* was obtained by PCR amplification of a 2,066-bp fragment from *E. coli* DH5α(λ*pir*) chromosomal DNA by using primers PrhaB-UP (5′-CGACCATGGCATATGTGATGGTGCTGAATTTCA) and PrhaB-DN (5′-CATATGCATTTAATCTTTCTGCGAATTGAGATG). The resulting fragment was then cloned into pGEM-T Easy (Promega, Madison, WI), which yielded pPS2036. The integrities of all cloned promoter-containing fragments were verified by DNA sequencing.

Flp and Cre excision of chromosomally integrated antibiotic resistance markers. Marker excision was performed using a previously described protocol (18) with minor modifications, as described below, for excision of a Zeo^r marker by use of either pFLPe4 or pCRE5. Briefly, Zeo^r strains were electroporated with ~150 ng of either pFLPe4 or pCRE5. Two aliquots (50 and 100 μl) and the concentrated rest of the mixture were plated on three LB plates containing 1,000 μg ml^{-1} Km (LB-Km1000) and incubated at 30°C for up to 48 h or until colonies were grown. Four Km^r transformants were struck for single colonies onto a four-sectored plate with LB-Km1000 plus 0.2% L-rhamnose, and the plate was incubated for 2 days at 30°C or until colonies were clearly discernible. Five colonies from each plate sector were then patched onto an LB-Km1000 plate and an LSLB plate containing 2,000 μg ml^{-1} Zeo (LSLB-Zeo2000), and the plates were incubated at 30°C for up to 48 h. Zeo-susceptible and Km^r cells were selected and streaked onto two or three LB plates for single colonies, and the plates were incubated at 42°C for 2 days or until colonies were discernible. Single colonies were tested at 30°C for susceptibility to Km and Zeo by patching them onto LB-Km1000, LSLB-Zeo2000, and LB plates. Km- and Zeo-susceptible cells were retained, and Flp or Cre-mediated marker excision was verified by colony PCR with *ble*-specific primers. The same protocol was used for Km^r marker excision with pFLPe2 and pCRE3, except that plasmid selection and recombinase induction steps were performed using LSLB-Zeo2000 plates. Rhamnose-inducible Flp and Cre gene expression can also be achieved in LB medium supplemented with 0.2% rhamnose and the appropriate antibiotics.

Isolation of an unmarked Δ(*amrRAB-oprA*) deletion by PCR fragment transformation and Flp excision. Gene disruption by deletion was performed using a previously described method (20). Briefly, using 5 to 50 ng of chromosomal or plasmid DNA templates, three partially overlapping DNA fragments representing flanking DNA segments and the Zeo^r marker from pFZE1 were amplified separately and then spliced together by an overlap extension PCR. To do this, the following fragments were amplified in a first-round PCR using the following primers: a 892-bp *amrR* upstream fragment using primers amrRAB-oprA-UP-For (5′-agggtgtccacatccttgaa) and amrRAB-oprA-UP-Rev-GM (5′-TCAGAGCGCTTTTGAAGCTAATTCGgacacttcaacggcaagat), a 828-bp *oprA* downstream fragment using amrRAB-oprA-DN-For-GM (5′-AGGAACTTCAAGATCCCCAATTCGgtcgccgaatacgagaagac) and amrRAB-oprA-DN-Rev (5′-gaaatacgccttgacgcact), and a 774-bp *ble* fragment using GmFRT-UP (5′-CGAATTAGCTTCAAAAGCGCTCTGA) and GmFRT-DN (5′-CGAATTGGGGATCTTGAAGTACCT). (Lowercase letters denote chromosome-specific sequences and uppercase letters *FRT* cassette-specific sequences.) These fragments were combined in a second PCR, and after gel purification, the resulting recombinant ~2.45-kb DNA fragment was cloned into pGEM-T Easy, which yielded pPS2254. The plasmid-borne deletion was transferred to the *B. pseudomallei* 1026b chromosome by transformation of competent cells, using an unpublished procedure developed in Colin Manoil's laboratory at the University of Washington (Thongdee et al., presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand). Briefly, strain 1026b cells were grown in DM medium to an optical density at 600 nm of 0.5. Cells from 1 ml of culture were harvested by centrifugation, and the cell pellet was resuspended in 50 μl of DM medium. One hundred nanograms of pPS2254 insert DNA purified from an agarose gel was added to the concentrated cells, and the mixture was incubated without shaking for 30 min at room temperature. After addition of 2 ml DM medium, the cell suspension was incubated with shaking at 30°C for 24 h. Cells were harvested by centrifugation, washed once with 1 ml DM medium, and then resuspended in 0.25 ml of fresh DM medium. Fifty-microliter aliquots were then plated on LSLB-Zeo2000 plates, and the plates were incubated at 37°C for 48 h. Deletion of the *amrR-amrAB-oprA* genes was confirmed by colony PCR using a previously described procedure (18). Finally, an unmarked Δ(*amrRAB-oprA*)::*FRT* deletion mutant was obtained by Flp-mediated excision of the Zeo^r marker, using pFLPe4. The presence of Δ(*amrRAB-oprA*)::*FRT* was verified using colony PCR and primers amrRAB-oprA-UP-For and amrRAB-oprA-DN-Rev.

Transfer of mutations between *B. pseudomallei* chromosomes. For transfer of antibiotic resistance-marked mutations, chromosomal DNA fragments (20 to 30 kb) were isolated using the QIAamp DNA mini kit, and the DNA was suspended in 200 μl of buffer AE (10 mM Tris-HCl, 0.5 M EDTA, pH 9). Approximately 150 ng of this DNA was added to competent cells, and bacteria were treated and plated as described above for recombinant DNA fragment transfer.

Site-specific transposition of mini-Tn*7* elements. Mini-Tn7 elements were transposed into the *B. thailandensis* and *B. pseudomallei* chromosomes, following the protocol previously described for *Burkholderia mallei*, using tri-parental mating for suicide vector delivery (14) and the helper plasmid pTNS3. pTNS3 was constructed by ligating a 375-bp EcoRI-SmaI fragment from pPS2225 between the same sites of pTNS2 (15). Insertion events were verified by colony PCR as previously described (14), utilizing the following primers. For *B. thailandensis*, the two PCR primers glmS1-DN (5′-GTTCGTCGTCCACTGGGATCA) and glmS2-DN (5′-AGATCGGATGGAATTCGTGGAG) were used to determine Tn*7* insertions downstream of either *glmS1* or *glmS2* in combination with primer Tn*7*L (P_{Tn7L}; 5′-ATTAGCTTACGACGCTACACCC). For *B. pseudomallei*, insertions at the *glmS1*, *glmS2*, and *glmS3* linked insertion sites were verified utilizing primers BPGLMS1 (5′-GAGGAGTGGGCGTCGATCAAC), BPGLMS2 (5′-ACACGACGCAAGAGCGGAATC), and BPGLMS3 (5′-CGG

ACAGGTTCGCGCCATGC) in combination with P_{Tn7L}. PCRs for *B. thailandensis* consisted of an initial denaturation step at 95°C for 2 min and then 30 cycles of 95°C for 45 s, 56°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The PCR cycle conditions were the same for *B. pseudomallei* except that the annealing temperature was 61°C.

Nucleotide sequence accession numbers. The plasmid sequences determined in this study were deposited in GenBank and assigned the following accession numbers: for pCRE3, EU215439; for pCRE5, EU215440; for pFGM1, AY597270; for pFKM2, EU215433; for pFLPe2, EU215437; for pFLPe4, EU215438; for pFRT2, AY597269; for pFRT3, EU240901; for pFTP1, AY712951; for pFZE1, DQ124230; for pLOX1, EU215436; for pLKM1, EU215434; for pLZE1, EU215435; for pTNS2, AY884833; for pTNS3, EU215432; for pUC18T-mini-Tn*7*T, AY599230; for pUC18T-mini-Tn*7*T-LAC, EU240902; for pUC18T-mini-Tn*7*T-Km-*FRT*, EU223384; for pUC18T-mini-Tn*7*T-Zeo-*FRT*, DQ493876; for pUC18T-mini-Tn*7*T-Km-*lox*, EU215442; for pUC18T-mini-Tn*7*T-Zeo-*lox*, EU215441; and for pUCP28T, U33751.

RESULTS

Isolation and copy number of a TS pRO1600 replicon. Plasmids based on the pRO1600 replicon (a cryptic plasmid isolated from a *P. aeruginosa* strain [41]) have widely been used for cloning with *B. pseudomallei* (9, 53). A TS pRO1600 replicon (hereafter named Ts_{Pa}) has previously been described for *Pseudomonas aeruginosa* (19). Because the wild-type pRO1600 replicon functions in *B. pseudomallei* (53) as well as in *B. mallei* and *B. thailandensis*, we reasoned that the previously isolated pRO1600(Ts_{Pa}) replicon would conditionally replicate in *Burkholderia* spp. To this end, the Ts_{Pa} replicon was used to replace the wild-type pRO1600 replicon from pUCP28T (53). The resulting plasmid, along with pUCP28T, was then used to transform *B. thailandensis*, and Tp^r transformants were selected at 30°C. In contrast to what was found for pUCP28T, which yielded numerous Tp^r colonies when transformed into *B. thailandensis*, no transformants were obtained with the pUCP28T(Ts_{Pa}) plasmid, despite numerous attempts. When transformed into *P. aeruginosa*, both plasmids yielded equal numbers of Tp^r transformants. These results indicated that the Ts_{Pa} replicon was nonreplicative in *B. thailandensis*, even at the permissive temperature.

We therefore decided to isolate a new pRO1600(Ts) replicon. To do this, the wild-type replicon (consisting of ori_{1600} and the *rep* coding sequence) was mutagenized by error-prone PCR, and a plasmid pool containing the cloned, mutagenized PCR fragments was directly transformed into *B. thailandensis* for screening of the TS phenotype. TS replicon mutants were obtained at a frequency of ~8%. Five representative TS plasmids were retained and their ori_{1600}-*rep* sequences determined. Compared to the wild-type ori_{1600}-*rep* sequence, all plasmids contained several mutations in the ori_{1600} or *rep* sequence. For example, TS replicon no. 1, which was retained for further studies, contained one mutation in the ori_{1600} region (an A-to-T change at nucleotide 986 of the published sequence; GenBank accession no. L30112) (60) and eight mutations in the *rep* gene sequence. Of the latter, three mutations did not change the Rep protein amino acid sequence, whereas five other mutations led to R19C, C31S, D145G, M157T, and G245R amino acid changes. We do not know whether all of these mutations are required for the observed TS phenotype. Intriguingly, the TS plasmids isolated in *B. thailandensis* (hereafter named Ts_{Bt}) replicated in *P. aeruginosa* at 30°C and did not exhibit a TS phenotype. Plasmid stability at 30°C was not assessed.

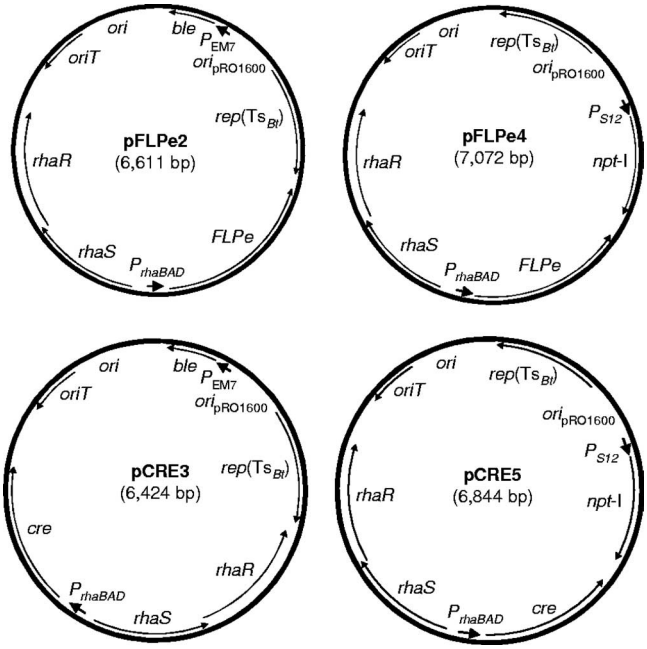

FIG. 1. Curable Flp and Cre recombinase-expressing plasmids. The plasmids contain the following shared features: *ori*, the *E. coli* pMB9 origin of replication; ori_{1600}, the pRO1600 origin of replication, requiring the *rep*(Ts_{Bt})-encoded replication protein, which confers a TS phenotype in *Burkholderia* spp. at temperatures above 37°C; *oriT*, an RK2-derived origin for conjugal plasmid transfer; and P_{rhaBAD}, the rhamnose-inducible *E. coli rhaBAD* operon promoter controlled by the *rhaR*- and *rhaS*-encoded regulatory proteins. Features unique to individual plasmids include *ble*, the bleomycin resistance gene, which confers Zeo resistance; *cre*, the Cre recombinase structural gene; *FLPe*, a gene encoding an enhanced Flp recombinase which is active over a larger temperature range than the wild-type enzyme; *nptI*, the neomycin phosphotransferase I gene, which confers Km resistance; P_{EM7}, a synthetic prokaryotic promoter; and P_{S12}, the promoter for the *B. thailandensis* ribosomal S12 protein-encoding gene.

Plasmids containing the Ts_{Bt} replicon were efficiently (>99.9%) cured from cells grown at either 37 or 42°C. Their copy numbers were ~1 (0.86 ± 0.23) in *B. thailandensis* cells grown at 30°C. By comparison, the copy numbers of plasmids containing the wild-type pRO1600 replicon were 3 to 4 (3.6 ± 0.85) in cells grown at the same temperature.

Construction of Flp and Cre recombinase-producing plasmids. Previous experience with a broad-host-range *P. aeruginosa* Flp-*FRT* system indicated that when Flp recombinase was transiently expressed from a nonreplicative plasmid, excision of the *FRT* cassette was observed only at very low frequencies (0.5 to 1%) (50). In contrast, excision frequencies were >99.9% when Flp recombinase was expressed from a replicative plasmid which could be cured from the cells after marker excision by *sacB*-mediated sucrose counterselection (28). Because *Burkholderia* spp. contain endogenous *sacB* genes (40), this previously developed broad-host-range system could unfortunately not easily be adapted for use with *Burkholderia* spp.

We therefore used the newly developed Ts_{Bt} replicon to derive curable plasmids producing Flp and Cre recombinases. The two most useful Flp-producing plasmids, pFLPe2 and pFLPe4 (Fig. 1), possess several desirable features: (i) they produce an Flp recombinase (Flpe) with enhanced recombi-

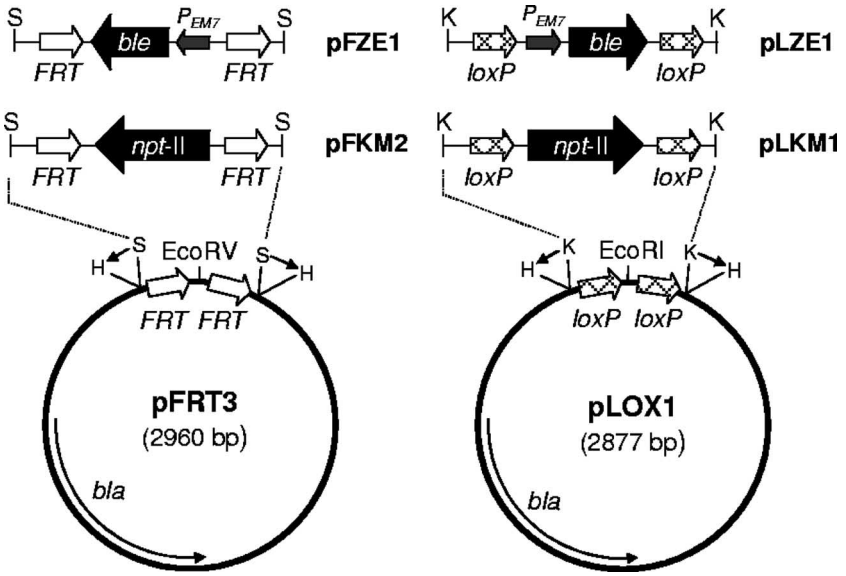

FIG. 2. Maps of *FRT* and *loxP* cassette vectors. Plasmid pFRT3 was derived from pFRT2 by fill-in of the unique NdeI site in the plasmid backbone. pLOX1 is based on the pFRT3 backbone and contains the pACD4K-C-loxP (Sigma-Aldrich)-engineered 5′ (CTACTTCGTATAGC ATACATTATACGAAGTTAT) *loxP* and 3′ (ATAACTTCGTATAGCATACATTATACGAAGTTAT) *loxP* sites, which show increased recombination efficiencies. The unique EcoRV and EcoRI sites are used for insertion of antibiotic resistance cassettes. Plasmids pFKM2 and pFZE1 were obtained by insertion of the neomycin phosphotransferase II (*nptII*) or Zeo binding protein (*ble*) gene into pFRT3 or pFRT2, respectively (see Table 1 for details). The same genes were inserted into the blunt-ended EcoRI site of pLOX1 to obtain pLKM1 and pLZE1. Abbreviations: *bla*, Ap resistance (β-lactamase) gene; P_{EM7}, synthetic prokaryotic promoter; S→H, SacI-KpnI-SmaI-BamHI-XbaI-SalI-PstI-SphI-HindIII; K→H, KpnI-SmaI-BamHI-XbaI-SalI-PstI-SphI-HindIII.

nase activity over a wider temperature range (5); (ii) *FLPe* gene expression is under the control of the rhamnose-regulated P_{rhaBAD} promoter, which was previously used for regulated gene expression in *Burkholderia cenocepacia* (8), and we reasoned that it could be used for the same purpose with other *Burkholderia* spp; (iii) they contain either a Km or a Zeo selection marker so that they are compatible with currently approved *B. pseudomallei* and *B. mallei* markers present on *FRT* cassettes; (iv) the *ble* and *nptI* genes are transcribed from the constitutive P_{EM7} and P_{S12} promoters, respectively, which enable expression of these genes in *Burkholderia* spp. (P_{EM7} is a synthetic bacterial promoter derived from the T7 promoter [Invitrogen]); (v) the plasmids contain an origin of conjugal transfer (*oriT*) which allows efficient introduction into strains or species recalcitrant to commonly used transformation methods; and (vi) the combination of low copy number (~1 per cell) and the Ts_{Bt} replicon allows for efficient plasmid curing.

Plasmids pFLPe2 and pFLPe4 allowed excision of Km and Zeo *FRT* gene cassettes with efficiencies of between 75 and 96% for pFLPe2 in *B. thailandensis*, 65% for pFLPe2, and 91% for pFLPe4 in *B. pseudomallei*. For an unknown reason, *B. thailandensis* cells were routinely difficult to transform with *nptI* containing pFLPe4 but easier with *nptII* containing pFLPe5.

To further extend the usefulness of site-specific excision technology with *Burkholderia* spp., we also constructed two Cre recombinase-producing plasmids, pCRE3 and pCRE5 (Fig. 1). With the exception of encoding Cre recombinase, these two plasmids possess the same useful features as those outlined above for the pFLPe plasmids. Diverse experiments demonstrated that Cre recombinase production was rhamnose inducible and that Cre-mediated site-specific excision was extremely efficient. Cre-mediated excision efficiencies in *B. thailandensis* were quite often significant, even in uninduced cells (ranging from 4 to 93%), and quite variable in rhamnose-induced cells (18 to 89%). Excision frequencies with pCRE5 were generally higher than those with pCRE3, approaching 90% in *B. pseudomallei*.

Excision efficiencies are probably not as high as those previously observed in *P. aeruginosa* (where they routinely approach 100%), because despite the high Zeo and Km concentrations used in these studies, we routinely found that not all colonies growing on the selection plates were true transformants but rather were antibiotic-tolerant cells which did not contain Cre- or Flp-producing plasmids.

Versatile Km, Tp, and Zeo resistance *FRT* and *loxP* cassettes. The pFLPe vectors described in this study can be used with *FRT* Gm, Tp, and Zeo resistance cassettes contained on pFGM1, pFTP1, and pFZE1 (Table 1). To complement these cassettes and maximize the applications of the newly established Cre site-specific recombination systems, a number of new cassette vectors were constructed (Fig. 2). Plasmid pFKM2 contains a Km^r *FRT* cassette, and pLKM1 and pLZE1 contain Km^r and Zeo^r *loxP* cassettes. For subcloning into other plasmids or transposons or for insertional mutagenesis, individual markers can easily be excised from the respective plasmids by using unique restriction sites in the flanking polylinkers. Alternatively, because their priming sites are conserved on all plasmids, the GmFRT-UP and GmFRT-DN primers can be utilized to PCR amplify the individual markers from the respective *FRT* plasmids, and similar strategies can be employed for amplification of *loxP* resistance cassettes.

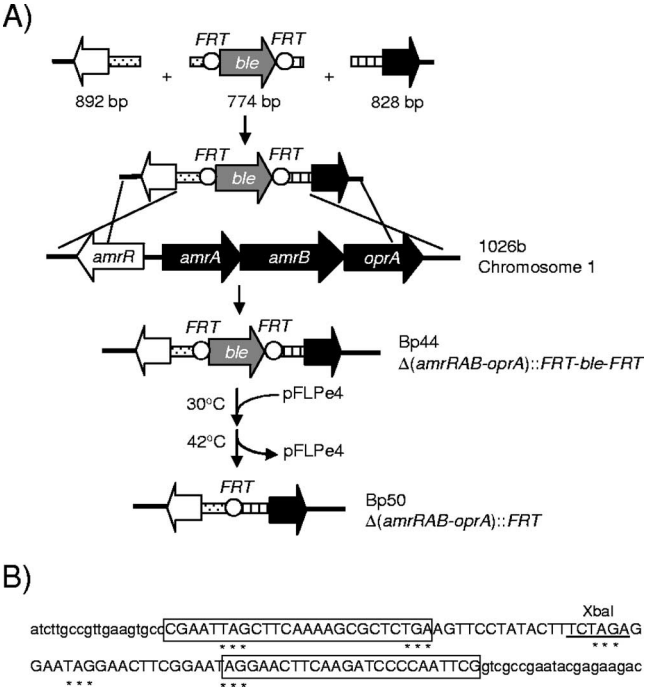

FIG. 3. Construction of an unmarked Δ(*amrRAB-oprA*) *B. pseudomallei* mutant. (A) Three partially overlapping DNA fragments were PCR amplified in separate reactions and then used as DNA templates in a second PCR to obtain a recombinant DNA fragment containing a *ble*-encoded Zeo resistance marker flanked by *FRT* sites as well as *amrR* and *oprA* 3′ sequences. This ~2.45-kb fragment was first cloned into pGEM-T Easy, excised from the plasmid, gel purified, and then introduced into strain 1026b by natural transformation, and Zeo-resistant transformants were selected. For excision of the Zeo resistance marker, the resulting strain, Bp44, was transformed with pFLPe4, followed by plating on rhamnose-containing media at 30°C to derive the unmarked strain Bp50. Plasmid pFLPe4 was then cured by culturing Zeo-susceptible cells at 42°C. (B) Sequence of the *FRT* scar region present in Bp50. Capital letters indicate the 86-bp *FRT* sequences. An XbaI site located within the *FRT* site is underlined, and GmFRT-UP and GmFRT-DN priming sites are boxed. The specific *amrR* and *oprA* priming sites are shown in lowercase letters. Asterisks mark potential translational stop codons carried by *FRT* sequences.

Isolation of a *B. pseudomallei* Δ(*amrRAB-oprA*) deletion. To assess the functionality of the newly developed Flp-*FRT* recombination system in *B. pseudomallei*, we decided to delete the *amrRAB-oprA* genes from strain 1026b. To this end, a Δ(*amrRAB-oprA*)::*FRT-ble-FRT* fragment was derived by splice overlap extension (SOE) PCR as illustrated in Fig. 3A and was cloned into pGEM-T Easy. For transfer of the plasmid-borne deletion, the Δ(*amrRAB-oprA*)::*FRT-ble-FRT* fragment was gel purified and transferred to the *B. pseudomallei* 1026b chromosome by transformation of competent cells. Finally, the unmarked Δ(*amrRAB-oprA*)::*FRT* deletion strain Bp50 was obtained by Flp-mediated excision of the Zeo^r marker (*ble*), using pFLPe4. The presence of the Δ(*amrRAB-oprA*)::*FRT-ble-FRT* and Δ(*amrRAB-oprA*)::*FRT* mutation in the respective strains was verified using colony PCR, which yielded the expected 2.45- and 1.76-kb fragments. The Δ(*amrRAB-oprA*)::*FRT* mutant Bp50 had deleted 49 of the 223 codons of *amrR*, the entire *amrA* and *amrB* genes, and 406 of the 513 codons of *oprA*. Flp-mediated excision was shown to leave behind a scar of 86 nucleotides (Fig. 3B). The Δ(*amrRAB-oprA*)::*FRT* mutant exhibited the same drug resistance phenotypes as the previously isolated Δ(*amrRAB-oprA*) *B. pseudomallei* strain DD503 (39) (Table 2).

Transfer of Δ(*amrRAB-oprA*) between *B. pseudomallei* strains. As there is no transduction system for *B. pseudomallei*, we explored natural transformation for transfer of mutations marked with antibiotic genes between different strains. To this end, 20- to 30-kb chromosomal DNA fragments from Δ(*amrRAB-oprA*)::*FRT-ble-FRT* strain Bp44 were used to transform strains 1026b and 1710b. Zeo^r 1026b transformants were obtained at a frequency of about 180 colonies per μg of DNA. The same procedure yielded fewer transformants with 1710b. Of six Zeo^r 1026b and 1710b colonies analyzed by PCR, four 1026b transformants and one 1710b transformant contained the *ble* gene. The five *ble*-containing colonies showed the same antibiotic resistance profile as the parental Bp44 strain and thus contained the Δ(*amrRAB-oprA*) deletion.

Establishment of a mini-Tn*7*-based single-copy gene integration system. Mini-Tn*7* elements are inserted into bacterial chromosomes at defined *att*Tn*7* sites (15, 43). With few exceptions (17), most of these sites lie in a neutral, intergenic region downstream of the essential *glmS* gene encoding glutamine-6-phosphate synthase. *Burkholderia* spp. are unique in that they contain multiple *glmS* genes and thus multiple *att*Tn*7* sites (14, 15), but insertions at the different sites can easily be distinguished by PCR utilizing insertion site-specific primers.

We previously described conjugation strategies for mini-Tn*7* delivery into *B. thailandensis* and *B. mallei* by use of helper plasmid pTNS2 (14, 15). To increase mini-Tn*7* transposition efficiencies in *Burkholderia* spp., pTNS3, in which the *tnsABCD*

TABLE 2. AmrAB-OprA efflux pump activity in *B. pseudomallei* 1026b and its mutants

Strain	MIC (μg/ml) of[b]:							
	IPTG[a]	Gm	Km	Ery	Cb	Cm	Nfx	Tet
1026b	NA	256	128	256	512	4	4	0.5
DD503	NA	1	4	8	512	4	4	1
Bp50	NA	2	4	8	512	4	4	0.25
Bp50::mini-Tn*7*T-LAC	None	2	4	8	512	4	4	0.25
Bp50::mini-Tn*7*T-LAC	+	2	4	8	512	4	4	0.25
Bp50::mini-Tn*7*T-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$	None	32	8	8	512	4	4	0.25
Bp50::mini-Tn*7*T-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$	+	1,024	128	256	512	4	4	0.5

[a] NA, not applicable; +, addition of IPTG to give a final concentration of 1 mM.
[b] Ery, erythromycin; Cb, carbenicillin, Cm, chloramphenicol; Nfx, norfloxacin; Tet, tetracycline.

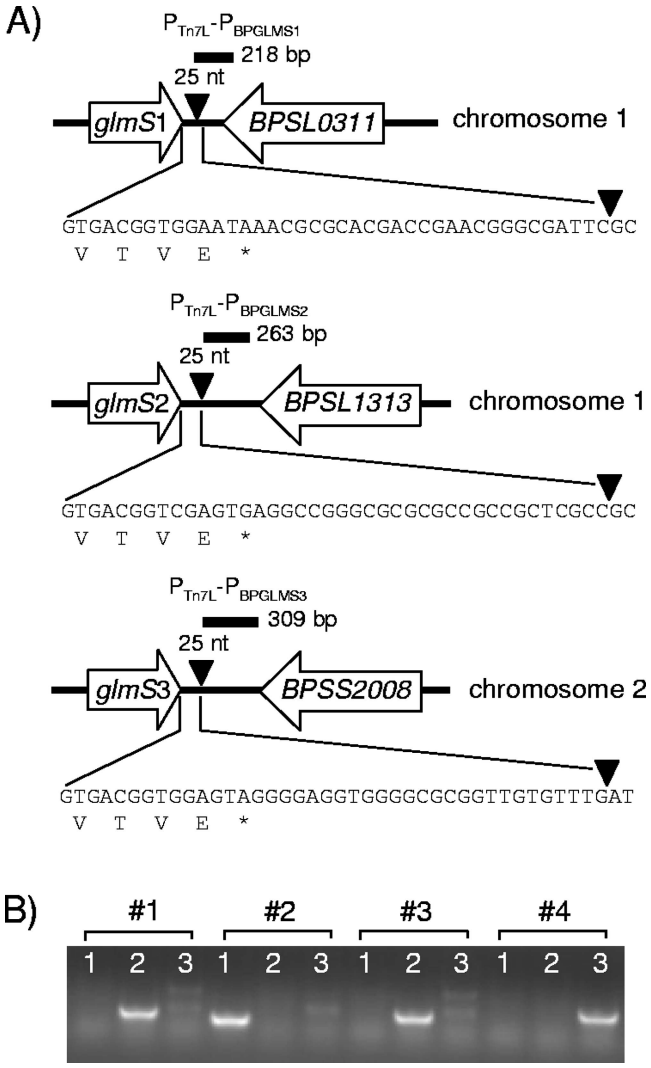

FIG. 4. Site-specific Tn*7* insertion in *B. pseudomallei*. (A) The insertion sites in K96243 were derived by PCR amplification of Tn*7*-chromosomal DNA junction sequences by using P_{Tn7L} and *glmS1*-, *glmS2*-, and *glmS3*-specific primers. Transposon-chromosomal junction DNA sequences were determined by sequencing of the PCR fragments (indicated by horizontal bars and labeled with the expected product sizes) amplified with the indicated primer pairs, taking into account the 5-bp duplication generated upon Tn*7* transposition. *BPSL0311*, *BPSL1313*, and *BPSS2008* are *B. pseudomallei* K96243 genes that encode proteins of unknown functions. Note that although these insertion sites are identical in other *B. pseudomallei* strains examined in this study, annotation numbers of the downstream genes are different (e.g., for strain 1026b) or not yet available at the time of these studies. Boxed arrows mark genes and their transcriptional orientations. The sequences shown encompass the last five codons of the respective *glmS* genes and their immediate downstream sequences, including the Tn*7* insertion sites, which are marked by triangles. (B) PCR verification of mini-Tn*7* insertion in strain 1026b at *glmS1* (1), *glmS2* (2), and *glmS3* (3) by use of the primer pairs shown in panel A. In this particular example, insertion no. 2 is at *glmS1*, insertions no. 1 and no. 3 are at *glmS2*, and insertion no. 4 is at *glmS3*. nt, nucleotides.

genes, encoding the site-specific Tn*7* transposition pathway, are transcribed from tandem promoters, the P1 integron promoter (*P1*) and the *E. coli lac* operon promoter (P_{lac}), was constructed. Mini-Tn*7* transposition efficiencies with pTNS3 in conjugation transfer experiments (14) were substantially increased to several thousand colonies per plate, compared to those observed with pTNS2, which yielded several hundred colonies per plate.

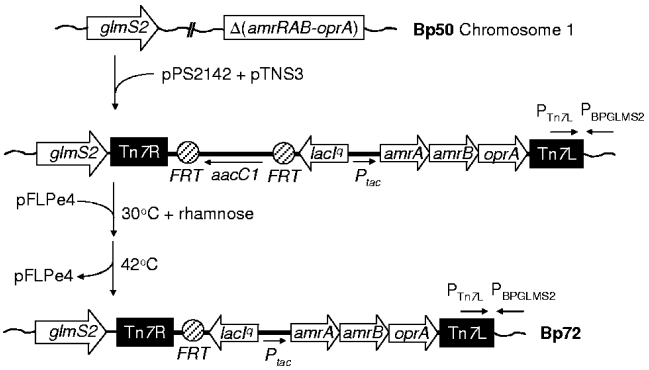

FIG. 5. Single-copy gene complementation of a *B. pseudomallei* Δ(*amrRAB-oprA*) deletion mutant. The suicide mini-Tn*7* delivery plasmid pPS2142 and helper plasmid pTNS3 were introduced into Δ(*amrRAB-oprA*)::*FRT* strain Bp50 by conjugation, followed by selection of transformants that were Gm resistant due to the *aacC1* gene present on the mini-Tn*7* element. In this strain, the 1026b *amrAB-oprA* operon is transcribed from the *tac* promoter (P_{tac}), which is under the control of the *lacI*-encoded *E. coli* Lac repressor. The Gm marker flanked by *FRT* sites was then excised using Flp recombinase, resulting in the unmarked *amrAB-oprA*-expressing strain Bp72. The negative-control strain Bp73 had the empty mini-Tn*7*T-LAC vector inserted at the same site on chromosome 1. Insertion at *glmS2* on chromosome 1 was verified by PCR using the primer pair P_{Tn7L} and $P_{BPGLMS2}$.

Utilizing pTNS3, a mini-Tn*7* element with a Zeo^r marker was inserted into the chromosome of *B. pseudomallei* 1026b, and insertion sites were determined utilizing primers BPGLMS1, BPGLMS2, and BPGLMS3, designed to yield PCR fragments of 218, 263, and 309 bp, respectively (Fig. 4). Insertions were readily obtained at all sites, although in >65% of all instances, the mini-Tn*7* elements transposed to the *glmS2* *att*Tn*7* site on chromosome 1. There was no obvious preference for either of the other two sites in the remainder of the transformants. Some transformants contained insertions at more than one *att*Tn*7* site, but while double insertions in two separate *att*Tn*7* sites were fairly common (10 to 20% with some strains), triple insertions were rarely observed. Sequence analysis revealed that all three *att*Tn*7* sites were located 25 bp downstream of the respective *glmS* genes. It should be noted that the C-terminal sequences of *B. pseudomallei glmS2* and its immediate downstream sequences differ from those of *B. mallei glmS1* and its immediate downstream sequences, the preferred mini-Tn*7* insertion site in *B. mallei*, by only a single nucleotide. The same insertion sites and site preferences were observed when mini-Tn*7* elements were transposed into strains K96243 and 08 (data not shown).

Single-copy complementation of a *B. pseudomallei* Δ(*amrRAB-oprA*) strain. To assess the utility of mini-Tn*7* elements for single-copy gene complementation in *B. pseudomallei*, a mini-Tn*7* element expressing the 1026b wild-type *amrAB-oprA* operon from the Lac repressor-regulated P_{tac} promoter was transposed to the chromosomes of Δ(*amrRAB-oprA*) strain Bp50 (Fig. 5). Since Bp50 is Gm susceptible, mini-Tn*7*-containing transformants could be selected on Gm-containing selective medium. As ex-

pected, mini-Tn*7* insertions were observed at all three *att*Tn*7* sites, with the majority occurring downstream of *glmS2* on chromosome 1, the same chromosome harboring the Δ(*amrRAB-oprA*) mutation. We selected transformants that had mini-Tn*7*-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$ integrated at *glmS2* or *glmS3* (for unknown reasons, we were unable to obtain insertions at *glmS1* in this particular experiment). AmrAB-OprA efflux pump activity was restored to wild-type levels in all strains, irrespective of the location of the complementing *amrA*$^+$*B*$^+$-*oprA*$^+$ operon, but only when *amrAB-oprA* gene expression was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to the growth medium (Table 2).

DISCUSSION

At the time of writing of this paper, only Gm, Km, and Zeo markers are approved for genetic manipulation of *B. pseudomallei*. Of these, only Km and Zeo can be used with wild-type *B. pseudomallei*, but only at concentrations exceeding 1,000 μg ml^{-1} (interestingly, only 100 μg ml^{-1} Zeo is required for clean selection in *B. thailandensis*). In *B. pseudomallei*, the use of Gm is restricted to efflux pump-deficient mutant derivatives. To preserve and extend the utility of the few selection markers currently available, we engineered site-specific Flp-*FRT* and Cre-*loxP* recombination systems for use with *Burkholderia* spp. All vectors are equipped with approved selection markers. The *nptI* and *nptII* genes employed in this study are also known as *aphA*(3′)-*I* and *aphA*(3′)-*II* and encode 3′ phosphotransferases (59). The products of these genes confer resistance to Km and neomycin but not to Gm and other clinically significant aminoglycosides (59). Similarly, the *Streptoalloteichus hindustanus ble* gene (26) encodes resistance to clinically insignificant antibiotics only, such as bleomycin and Zeo. The Flp-*FRT* and Cre-*loxP* recombination systems were carefully engineered to maximize their potential applications. For example, for excision of Zeo^r cassettes, pCRE5 and pFLPe4, with a Km selection marker, were constructed. Conversely, pCRE3 and pFLPe2 can be used for excision of Km^r markers. Either the pCRE or the pFLPe vector can be used for excision of markers other than Km or Zeo. Supporting these plasmids are *loxP* and *FRT* cassette vectors with Gm, Km, Tp, and Zeo resistance markers. It should be noted that in the United States, Tp is not approved for use with *B. mallei* and *B. pseudomallei*, and its use is therefore restricted to *B. thailandensis* and other *Burkholderia* spp. Additionally, according to select-agent guidelines, Gm cannot be used with *B. mallei*. The pLOX1 and pFRT3 cassette vectors are pUC-based high-copy-number plasmids containing unique EcoRI and EcoRV restriction sites flanked by two *loxP* or *FRT* sites. As additional approved selection markers become available, these vectors allow construction of new *loxP* or *FRT* resistance cassettes.

In bacteria such as *Burkholderia* spp. that exhibit high intrinsic resistance to many antimicrobials or where the use of a resistance marker is restricted by select-agent regulations, one can envision recycling the same marker for isolation of consecutive chromosomal mutations and/or extrachromosomal element insertions in the same strain. As previously discussed (28, 49), one possible drawback to such an approach is that each Cre or Flp excision event leaves behind a *loxP* or *FRT* site, which could lead to undesirable recombination events upon reintroduction of the respective site-specific recombinases, causing deletions or inversion of chromosomal segments. However, these are extremely rare (during the many instances in which we have used the Flp-*FRT* system with *P. aeruginosa*, we have observed a single large chromosomal inversion between recombination sites that were 1.6 Mb apart [2]) but may be more common when recombination sites are spaced closely together on the chromosome, e.g., within the same operon. Such undesirable recombination events can easily be detected by PCR across recombination sites, which should be performed as a matter of routine. The 33-bp *loxP* and 86-bp *FRT* scars left behind after Cre and Flp excision of our cassettes are not substrates for the bacterial recombination machinery. We anticipate that the *loxP* and *FRT* cassettes will be valuable tools for micro- and macromanipulations of entire *Burkholderia* spp. chromosomes.

Many bacterial genes are organized in operons, and when promoter-proximal genes are deleted in such instances, it is desirable to isolate unmarked in-frame deletions to avoid possible unwanted effects on distal genes. This is usually achieved by merodiploid formation, resolution to either the wild-type or a mutant copy by use of counterselection against unwanted plasmid sequences, and screening for wild-type versus mutant alleles by phenotype and/or PCR. Aside from relying on spontaneous merodiploid resolution, such strategies are not feasible with wild-type bacteria, because no reliable counterselection marker that works with wild-type *B. pseudomallei* currently exists. Although the use of *sacB*-mediated counterselection has been reported by a few groups (10, 24), it is not known whether the deletion events were due to *sacB*-mediated sucrose toxicity rather than to spontaneous resolution events. Routine use of *sacB*-based counterselection in the closely related species *B. mallei* is possible only after deletion of the chromosomal *sacB* gene (40), and *B. pseudomallei* strains contain chromosomal *sacB* genes with 99 to 100% amino acid sequence identity to *B. mallei sacB* (e.g., BPSS0543 in K96243 and BURPS1710b_A2102 in 1710b). However, a recent report indicates that *sacB*-mediated counterselection in some *B. pseudomallei* strains may be possible under certain conditions (C. Logue, I. R. Peak, and I. R. Beacham, presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand).

Even though Cre and Flp excision events leave behind scars, these methodologies can also be used for isolation of in-frame deletion mutants. Besides serving as recombination sites, these scars are benign, i.e., they encode no promoter or transcriptional terminator sequences. However, they do contain translational stop codons; one TAG codon is found in the *loxP* sequence (5′-CTACTTCGTATAGCATACATTATACGAA GTTAT), and five stop codons are found in two separate frames in the *FRT* scar (Fig. 3A). In some instances, therefore, translation will be terminated within the scar sequences. Additionally, the scar can easily be placed in-frame during PCR-mediated assembly of the mutagenic DNA fragment by designing the gene-specific portions of the SOE PCR primers accordingly.

The one-step DNA fragment mutagenesis method is a rapid method which does not require counterselection and thus has widespread utility in *Burkholderia* wild-type strains. However, it has some drawbacks compared to the traditional method using a nonreplicative suicide plasmid. First, mutated frag-

ments need to be tagged with an antibiotic resistance selection marker so that recombinational events can be selected. With the availability of Cre and Flp excision systems, however, this is not a serious impediment. Second, the method does not allow for transfer of single mutations, for example, TS alleles, into the chromosome without linking them to a selection marker, which, by definition, would introduce a secondary mutation. Such experiments will have to await development of counterselection methods which work with wild-type *Burkholderia* spp. Third, it may not work with all *B. pseudomallei* strains, e.g., K96243 (Thongdee et al., presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand). While we routinely use cloned and purified DNA fragments for mutagenesis, purified PCR products can be directly used for transformation (Thongdee et al., presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand).

In the absence of phage transduction, electroporation or natural transformation of bacteria with chromosomal DNA fragments containing a selectable marker is the only means of transferring mutations between chromosomes. A report in the Russian literature in the mid-1980s indicated that spontaneous transformation can be used to transfer DNA into and between *B. pseudomallei* strains (6), which was corroborated by recent observations (Thongdee et al., presented at the 5th World Melioidosis Congress, Khon Kaen, Thailand). We also demonstrated that natural transformation can be utilized to transfer antibiotic resistance-marked mutations between *B. pseudomallei* chromosomes, most efficiently within the same strain background but with reduced efficiency also between different strains. Natural transformation with linear fragments, as well as transfer of chromosomal DNA fragments, both containing a selectable marker, now allows combining of diverse mutations for rapid construction of isogenetic mutants, mutations in different strain backgrounds, etc. Unlike live strains, sterile DNA is exempt from select-agent rulings, and transfer between different laboratories does not require permits. "Mutants" can thus easily be shared between laboratories in the form of fragmented chromosomal DNA and reconstructed using linear fragment transfer, provided that the recipient uses an identical or compatible strain background and is approved for work with *B. pseudomallei*.

The unmarked Δ(*amrAB-oprA*) strains generated in this study allow utilization of the Gm^r marker for genetic manipulation in these strains, for example, complementation with mini-Tn*7* elements (this study). Wild-type *B. pseudomallei* strains are highly streptomycin resistant because this antibiotic is an AmrAB-OprA substrate. As Δ(*amrAB-oprA*) mutants are streptomycin susceptible (39), they allow use of streptomycin resistance markers for genetic manipulations. First, though to our knowledge they are currently not yet approved for use in *B. pseudomallei*, streptomycin resistance cassettes (45) could be utilized similarly to the Km and Zeo cassettes employed in this study. Second, spontaneous streptomycin-resistant *rpsL* mutant derivatives of the Δ(*amrAB-oprA*) strains would allow utilization of streptomycin-based counterselection (56), which has previously been possible with strain DD503 only (58). However, there is evidence that *B. pseudomallei rpsL* mutants are attenuated in a murine model of pneumonic melioidosis (30) but not in the murine inhalation and hamster models (58), and such mutants may thus not be suitable for pathogenesis studies involving certain animal models.

Although a previous report in the Russian literature indicated the use of an RP1 TS replicon in *B. pseudomallei* (36), these plasmids have to our knowledge not been characterized in molecular detail and are not readily available. These shortcomings are overcome by the newly described pRO1600(Ts) replicon. It allows for rapid curing of plasmids from bacterial cells and will facilitate development of other genetic tools for these bacteria, for example, more-efficient transposon mutagenesis systems. While we do not know which of the five amino acid substitutions of the ori_{1600} Rep protein are responsible for the TS phenotype, we have not yet observed spontaneous revertants, indicating that more than one mutation is needed to convey a TS phenotype. Plasmids containing either the wild-type or the TS ori_{1600} replicon are low copy number in *Burkholderia* spp., which makes them useful for cloning of genes whose products may be detrimental to host cells when the genes are cloned in higher-copy-number plasmids.

In many instances, development of genetic tools for any given bacterium is hampered by lack of suitable promoters for either constitutive or regulated gene expression. In this study, we successfully used the P1 integron and the synthetic bacterial P_{EM7} and *B. thailandensis* S12 ribosomal gene (P_{S12}) promoters for constitutive expression of selection markers in *Burkholderia* spp. Furthermore, we demonstrated the utility of the *E. coli rhaBAD* operon and *tac* promoters for regulated gene expression in *B. pseudomallei* and *B. thailandensis*. Although it is not shown in this study, we obtained evidence that the *E. coli araBAD* promoter can also be used for arabinose-regulated gene expression in *B. thailandensis* and, by extension, probably also in *B. pseudomallei*. In many instances, significant Cre-mediated marker excision was observed in uninduced cells carrying the pCRE5 plasmid, indicating that the P_{rhaBAD} promoter may be leaky in LSLB-grown cells. It has been reported that P_{rhaBAD} is responsive to low concentrations of rhamnose and can be effectively repressed with 0.2% glucose in *B. cenocepacia* (8), but we have not yet explored glucose repression in *B. pseudomallei*.

Construction of an improved transposase delivery and expression vector in conjunction with marker excision systems also enabled development of a mini-Tn*7*-based site-specific genome integration system for *B. pseudomallei*. The mini-Tn*7* system has many advantages compared to other integration systems: (i) it is easy and efficient to use, and detailed user protocols have been published (14, 17, 18); (ii) mini-Tn*7* elements can be readily transferred into different mutant backgrounds, thus facilitating comparative gene expression analyses; (iii) chromosomally integrated mini-Tn*7* elements replicate stably without continued antibiotic selection for at least 100 bacterial generations; and (iv) they are highly versatile and can be used instead of plasmids for gene cloning and complementation, regulated expression from the *E. coli tac* promoter, isolation of transcriptional or translational β-galactosidase (*lacZ*) fusions, transcriptional bacterial luciferase (*lux*) fusions, and tagging with green fluorescent protein or its cyan, yellow, and red variants (18). In comparison to other bacterial species, *Burkholderia* spp. possess multiple *glmS*-linked *att*Tn*7* sites. As indicated by the occasional occurrence of multiple insertions in the same transformant, multiple in-

sertion sites can be exploited to tag the same bacterium with different mini-Tn*7* elements, for example, a gene fusion tag and a complementing expression element, allowing gene expression and complementation analyses using single-copy constructs, as nature intended it. In this paper, we demonstrated the use of the mini-Tn*7* system for complementation of a Δ(*amrRAB-oprA*) mutant. For this purpose, we selected transformants that had a mini-Tn*7*-LAC-*amrA*$^+$*B*$^+$-*oprA*$^+$ expression vector integrated at *glmS2* or *glmS3*. AmrAB-OprA efflux pump activity was restored to wild-type levels, irrespective of the genomic location—chromosome 1 or chromosome 2—of the complementing *amrA*$^+$*B*$^+$-*oprA*$^+$ operon.

In summary, the genetic tools described in this paper allow routine select-agent-ruling-compliant manipulations of *B. pseudomallei* and thus facilitate much-needed studies of the biology and pathogenesis of this understudied yet fascinating bacterium. While they were not explicitly tested in this study, it can be anticipated that some of these tools will also be useful for studies of other *Burkholderia* spp., for example, *B. mallei*, for which similar select-agent restrictions apply. Though the methods described here were developed to allow compliance with guidelines in the United States, we also recommend their implementation in settings not governed by these rulings to avoid usage of selection markers encoding resistance to clinically significant antibiotics and to facilitate resource sharing.

ACKNOWLEDGMENTS

H.P.S. was supported by NIH NIAID grant U54 AI065357.

We are indebted to Metawee Thongdee and Colin Manoil (University of Washington) for providing pCre1 and the protocol for PCR fragment transformation and preparation of *Burkholderia* competent cells prior to publication. We thank Don Woods (University of Calgary) and Mark Schell (University of Georgia) for the gift of *B. pseudomallei* strains, Phoebe Rice (University of Chicago) for providing pET24d-FLPe, and Tung Hoang (University of Hawaii) for pointing out use of the S12 promoter for constitutive gene expression in *Burkholderia* spp.

REFERENCES

1. **Abremski, K., A. Wierzbicki, B. Frommer, and R. H. Hoess.** 1986. Bacteriophage P1 Cre-loxP site-specific recombination. Site-specific DNA topoisomerase activity of the Cre recombination protein. J. Biol. Chem. **261:**391–396.
2. **Barekzi, N., K. Beinlich, T. T. Hoang, X. Q. Pham, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2000. High-frequency Flp recombinase-mediated inversions of the *oriC*-containing region of the *Pseudomonas aeruginosa* genome. J. Bacteriol. **182:**7070–7074.
3. **Brent, A. J., P. C. Matthews, T. L. Pitt, and R. Handy.** 2007. Misdiagnosing melioidosis. Emerg. Infect. Dis. **13:**349–351.
4. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48:**317–320.
5. **Buchholz, F., P.-O. Angrand, and A. F. Stewart.** 1998. Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat. Biotechnol. **16:** 657–662.
6. **Bulantsev, A. L., and N. A. Lozovaia.** 1985. Spontaneous genetic transformation in *Pseudomonas pseudomallei*. Mol. Gen. Mikrobiol. Virusol. **1:** 11–15.
7. **Camilli, A., D. T. Beattie, and J. Mekalanos.** 1994. Use of genetic recombination as a reporter of gene expression. Proc. Natl. Acad. Sci. USA **91:**2634–2638.
8. **Cardona, S. T., and M. A. Valvano.** 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. Plasmid **54:**219–228.
9. **Chan, Y. Y., and K. L. Chua.** 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. J. Bacteriol. **187:**4707–4719.
10. **Chan, Y. Y., T. M. C. Tan, Y. M. Ong, and K. L. Chua.** 2004. BpeAB-OprB, a multidrug efflux pump in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. **48:**1128–1135.
11. **Chen, Y., and P. A. Rice.** 2003. The role of the conserved Trp330 in Flp-mediated recombination. Functional and structural analysis. J. Biol. Chem. **278:**24800–24807.
12. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.
13. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
14. **Choi, K.-H., D. DeShazer, and H. P. Schweizer.** 2006. mini-Tn*7* insertion in bacteria with multiple *glmS*-linked *att*Tn*7* sites: example *Burkholderia mallei* ATCC 23344. Nat. Protoc. **1:**162–169.
15. **Choi, K.-H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2005. A Tn*7*-based broad-range bacterial cloning and expression system. Nat. Methods **2:**443–448.
16. **Choi, K.-H., A. Kumar, and H. P. Schweizer.** 2006. A 10 min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64:**391–397.
17. **Choi, K.-H., and H. P. Schweizer.** 2006. mini-Tn*7* insertion in bacteria with secondary, non-*glmS*-linked *att*Tn*7* sites: example *Proteus mirabilis* HI4320. Nat. Protoc. **1:**170–178.
18. **Choi, K.-H., and H. P. Schweizer.** 2006. mini-Tn*7* insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa*. Nat. Protoc. **1:**153–161.
19. **Choi, K.-H., L. A. Trunck, A. Kumar, T. Mima, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2008. Genetic tools for *Pseudomonas*, p. 65–86. *In* P. Cornelis (ed.), *Pseudomonas* genomics and molecular biology. Calister Academic Press, Norfolk, United Kingdom.
20. **Choi, K.-H., and H. P. Schweizer.** 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiol. **5:**30.
21. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A7, 7th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
22. **DeShazer, D., P. Brett, R. Carlyon, and D. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.
23. **DeShazer, D., and D. E. Woods.** 1996. Broad-host-range cloning and cassette vectors based on the R388 trimethoprim resistance gene. BioTechniques **20:**762–764.
24. **Essex-Lopresti, A. E., J. A. Boddey, R. Thomas, M. P. Smith, M. G. Hartley, T. Atkins, N. F. Brown, C. H. Tsang, I. R. Peak, J. Hill, I. R. Beacham, and R. W. Titball.** 2005. A type IV pilin, PilA, contributes to adherence of *Burkholderia pseudomallei* and virulence in vivo. Infect. Immun. **73:**1260–1264.
25. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
26. **Gatignol, A., H. Durand, and G. Tiraby.** 1988. Bleomycin resistance conferred by a drug-binding protein. FEBS Lett. **230:**171–175.
27. **Gilbertson, L.** 2003. Cre-lox recombination: Cre-ative tools for plant biotechnology. Trends Biotechnol. **21:**550–555.
28. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
29. **Holden, M. T. G., R. W. Titball, S. J. Peacock, A. M. Cerdeno-Tarraga, T. P. Atkins, L. C. Crossman, T. L. Pitt, C. Churcher, K. L. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. F. Oyston, and J. Parkhill.** 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. USA **101:**14240–14245.
30. **Jeddeloh, J. A., D. L. Fritz, D. M. Waag, J. M. Hartings, and G. P. Andrews.** 2003. Biodefense-driven murine model of pneumonic melioidosis. Infect. Immun. **71:**584–587.
31. **Kite-Powell, A., J. R. Livengood, J. Suarez, R. Hopkins, T. A. Clark, and D. Chertow.** 2006. Imported melioidosis—South Florida, 2005. Morb. Mortal. Wkly. Rep. **55:**873–876.
32. **Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. De Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. **177:**52–58.
33. **Lee, C., J. Kim, S. G. Shin, and S. Hwang.** 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. **123:**273–280.
34. **Liss, L.** 1987. New M13 host: DH5αF′ competent cells. Focus **9:**13.

35. **Luo, H., and A. P. Kausch.** 2002. Application of FLP/FRT site-specific DNA recombination system in plants. Genet. Eng. (N.Y.) **24:**1–16.
36. **Merinova, L. K., E. V. Timofeeva, and I. K. Seimova.** 1997. Insertion mutations in *Pseudomonas pseudomallei* genome induced by transposons Tn*10*, Tn*9* and Tn*5*. Mol. Gen. Mikrobiol. Virusol. **9:**14–17.
37. **Metcalf, W. W., W. Jiang, L. L. Daniels, S.-K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZ*α for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1–13.
38. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Moore, R. A., D. DeShazer, S. Reckseidler, A. Weissman, and D. E. Woods.** 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. **43:**465–470.
40. **Nierman, W. C., D. DeShazer, H. S. Kim, H. Tettelin, K. E. Nelson, T. Feldblyum, R. L. Ulrich, C. M. Ronning, L. M. Brinkac, S. C. Daugherty, T. D. Davidsen, R. T. Deboy, G. Dimitrov, R. J. Dodson, A. S. Durkin, M. L. Gwinn, D. H. Haft, H. Khouri, J. F. Kolonay, R. Madupu, Y. Mohammoud, W. C. Nelson, D. Radune, C. M. Romero, S. Sarria, J. Selengut, C. Shamblin, S. A. Sullivan, O. White, Y. Yu, N. Zafar, L. Zhou, and C. M. Fraser.** 2004. Structural flexibility in the *Burkholderia mallei* genome. Proc. Natl. Acad. Sci. USA **101:**14246–14251.
41. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150:**60–69.
42. **Ow, D. W.** 2007. GM maize from site-specific recombination technology, what next? Curr. Opin. Biotechnol. **18:**115–120.
43. **Peters, J. E., and N. L. Craig.** 2001. Tn*7*: smarter than we thought. Nat. Rev. Mol. Cell Biol. **2:**806–814.
44. **Phillips, G. J.** 1999. New cloning vectors with temperature-sensitive replication. Plasmid **41:**78–81.
45. **Prentki, P., and H. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
46. **Rolim, D. B., D. C. F. L. Vilar, A. Q. Sousa, I. S. Miralles, D. C. A. de Oliveira, G. Harnett, L. O'Reilly, K. Howard, I. Sampson, and T. J. J. Inglis.** 2005. Melioidosis, northeastern Brazil. Emerg. Infect. Dis. **11:**1458–1460.
47. **Sadowski, P.** 1995. The Flp recombinase of the 2-μm plasmid of *Saccharomyces cerevisiae*. Prog. Nucleic Acid Res. Mol. Biol. **51:**53–91.
48. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. **Schweizer, H. P.** 2003. Applications of the *Saccharomyces cerevisiae* Flp/*FRT* system in bacterial genetics. J. Mol. Microbiol. Biotechnol. **5:**67–77.
50. **Schweizer, H. P.** 1998. Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: application of a novel technique for generation of unmarked chromosomal mutations for the study of efflux systems. Antimicrob. Agents Chemother. **42:**394–398.
51. **Schweizer, H. P.** 1994. A method for construction of bacterial hosts for *lac*-based cloning and expression vectors: a complementation and regulated expression. BioTechniques **17:**452–456.
52. **Schweizer, H. P., and V. de Lorenzo.** 2004. Molecular tools for genetic analysis of pseudomonads, p. 317–350. *In* J. L. Ramos (ed.), The pseudomonads: genomics, life style and molecular architecture, vol. I. Kluwer Academic/Plenum, New York, NY.
53. **Schweizer, H. P., T. R. Klassen, and T. Hoang.** 1996. Improved methods for gene analysis and expression in *Pseudomonas*, p. 229–237. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), Molecular biology of pseudomonads. ASM Press, Washington, DC.
54. **Sorrell, D. A., and A. F. Kolb.** 2005. Targeted modification of mammalian genomes. Biotechnol. Adv. **23:**431–469.
55. **Sternberg, N., and D. Hamilton.** 1981. Bacteriophage P1 site-specific recombination. I. Recombination between *loxP* sites. J. Mol. Biol. **150:**467–486.
56. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
57. **Theodosiou, N. A., and T. Xu.** 1998. Use of FLP/FRT system to study Drosophila development. Methods **14:**355–365.
58. **Ulrich, R. L., D. DeShazer, E. E. Brueggemann, H. B. Hines, P. C. Oyston, and J. A. Jeddeloh.** 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. J. Med. Microbiol. **53:**1053–1064.
59. **Vakulenko, S. B., and S. Mobashery.** 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. **16:**430–450.
60. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81–86.
61. **Wiersinga, W. J., T. van der Poll, N. J. White, N. P. Day, and S. J. Peacock.** 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nat. Rev. Microbiol. **4:**272–282.
62. **Yu, M., and J. S. Tsang.** 2006. Use of ribosomal promoters from *Burkholderia cenocepacia* and *Burkholderia cepacia* for improved expression of transporter protein in *Escherichia coli*. Protein Expr. Purif. **49:**219–227.
63. **Yu, Y., H. S. Kim, H. H. Chua, C. H. Lin, S. H. Sim, D. Lin, A. Derr, R. Engels, D. DeShazer, B. Birren, W. C. Nierman, and P. Tan.** 2006. Genomic patterns of pathogen evolution revealed by comparison of *Burkholderia pseudomallei*, the causative agent of melioidosis, to avirulent *Burkholderia thailandensis*. BMC Microbiol. **6:**46.