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Engineering of Tellurite-Resistant Genetic Tools for Single-Copy Chromosomal Analysis of *Burkholderia* spp. and Characterization of the *Burkholderia thailandensis betBA* Operon▽†

Yun Kang,[2] Michael H. Norris,[2] Ashley R. Barrett,[1] Bruce A. Wilcox,[3] and Tung T. Hoang[1,2]*

Department of Microbiology,[1] Department of Molecular Biosciences and Bioengineering,[2] and Department of Ecology and Health,[3] University of Hawaii at Manoa, Honolulu, Hawaii 96822

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There are few appropriate single-copy genetic tools for most *Burkholderia* species, and the high level of antibiotic resistance in this genus further complicates the development of genetic tools. In addition, the utilization of resistance genes for clinically important antibiotics is prohibited for the bioterrorism agents *Burkholderia pseudomallei* and *Burkholderia mallei*, necessitating the development of additional nonantibiotic-based genetic tools. Three single-copy systems devoid of antibiotic selection based on two nonantibiotic selectable markers, tellurite resistance (Tel^r) and *Escherichia coli* aspartate-semialdehyde dehydrogenase (asd_{Ec}), were developed to facilitate genetic manipulation in *Burkholderia* species. These systems include one mariner transposon, a mini-Tn*7*-derived site-specific transposon, and six *FRT* reporter fusion vectors based on the *lacZ*, *gfp*, and *luxCDABE* reporter genes. Initially, we showed that the random mariner transposon pBT20-Δ*bla*-Tel^r-*FRT* efficiently transposed within *Burkholderia cenocepacia*, *Burkholderia thailandensis*, *B. pseudomallei*, and *B. mallei*. We then utilized the mini-Tn*7*-Tel^r-based transposon vector (mini-Tn7-Tel^r-*betBA*) and a transposase-containing helper plasmid (pTNS3-asd_{Ec}) to complement the *B. thailandensis* Δ*betBA* mutation. Next, one of the *FRT-lacZ* fusion vectors (pFRT1-*lacZ*-Tel^r) was integrated by Flp (encoded on a helper plasmid, pCD13SK-Flp-*oriT*-asd_{Ec}) to construct the *B. thailandensis* Δ*betBA*::*FRT-lacZ*-Tel^r reporter fusion strain. The *betBA* operon was shown to be induced in the presence of choline and under osmotic stress conditions by performing β-galactosidase assays on the *B. thailandensis* Δ*betBA*::*FRT-lacZ*-Tel^r fusion strain. Finally, we engineered *B. thailandensis* Δ*betBA*::*FRT-gfp*-Tel^r and Δ*betBA*::*FRT-lux*-Tel^r fusion strains by utilizing fusion vectors pFRT1-*gfp*-Tel^r and pFRT1-*lux*-Tel^r, respectively. The induction of the *betBA* operon by choline and osmotic stress was confirmed by performing fluorescent microscopy and bioluminescent imaging analyses.

The genus *Burkholderia*, consisting of more than 40 different species, occupies diverse ecological niches ranging from the soil rhizosphere to the human respiratory tract (39). Within this genus, members exhibit considerable genetic diversity and broad metabolic capabilities (26, 39), facilitating their adaptation to a variety of environmental conditions including nutrient limitation, the presence of antibiotics and toxic compounds, and pH fluctuations. Many *Burkholderia* species are known plant pathogens, including *Burkholderia caryophylli*, *B. plantarri*, and *B. glumae*, while others (e.g., *B. cepacia* complex) cause opportunistic infections (39). In addition, *Burkholderia pseudomallei* and *B. mallei* are primary pathogens for humans and animals and are listed as category B select agents in the United States.

To best exploit the genomic information available for several *Burkholderia* species, a wide array of tools is required for molecular genetic and pathogenesis studies of these bacteria. For *Burkholderia* species not classified as select agents, antibiotic-resistance-based tools could be used for genetic manipulation. However, the Centers for Disease Control and Prevention restricts the introduction of markers conferring resistance against clinically important antibiotics into the two select agents *B. mallei* and *B. pseudomallei*. At present, only gentamicin, kanamycin, and zeocin resistance markers are approved for limited use for *B. pseudomallei*, while only the kanamycin and zeocin resistance markers are approved for *B. mallei* (35). However, most wild-type strains of *B. mallei* and *B. pseudomallei* have high levels of resistance to all three antibiotics (7, 29, 36), and even at high concentrations, the selection is not tight, and spontaneous resistance still arises (10, 15, 32). Consequently, there is still a need to expand universal genetic tools based on nonantibiotic selectable markers, allowing broader applications in various *Burkholderia* species.

Several nonantibiotic selection schemes have been used in bacteria including, but not limited to, resistance to various compounds (e.g., arsenate; bialaphos or its degradation product, phosphinothricin; mercury; and tellurite [Tel]) and metabolic markers (e.g., lactose utilization and purine and amino acid biosynthesis). Potential drawbacks to using arsenate and mercury are high toxicity levels and narrow selective concentration ranges (4, 16). Bialaphos and its degradation product, phosphinothricin, have been shown to be ineffective for *Burkholderia* select agents, requiring concentrations greater than 1,000 μg/ml, whereas these bacteria have been shown to be

* Corresponding author. Mailing address: Department of Microbiology, University of Hawaii at Manoa, 2538 The Mall-Snyder 310, Honolulu, HI 96822. Phone: (808) 956-3522. Fax: (808) 956-5339. E-mail: tongh@hawaii.edu.

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sensitive to Tel concentrations of less than 1 μg/ml (M. Frazier, K. Choi, A. Kumar, C. Lopez, R. R. Karkhoff-Schweizer, and H. P. Schweizer, presented at the American Society for Microbiology Biodefense and Emerging Diseases Research Meeting, Washington, DC, 2007). Therefore, the nonantibiotic selectable marker based on Tel resistance (Tel^r) could be useful for genetic manipulation in various *Burkholderia* species, particularly *B. mallei* and *B. pseudomallei*. The Tel^r marker, consisting of three genes (*kilA*, *telA*, and *telB*) (38), has been successfully employed as a nonantibiotic selectable marker originally in *Pseudomonas putida* (34), in several other gram-negative bacteria (25), and, more recently, in *B. thailandensis* (2). Additionally, the *asd* gene (a metabolic marker encoding aspartate-semialdehyde dehydrogenase for amino acid biosynthesis) has been used as a nonantibiotic selectable marker in Δ*asd* backgrounds (2, 30). Combining the Tel^r marker and the *asd* gene may expand the repertoire of genetic tools available for *Burkholderia* species.

Strategies and tools for the manipulation of genetic elements as a single copy on the chromosome have been developed, such as *Himar1*-based mariner transposons (22, 32), the mini-Tn*7* site-specific transposition system (1, 9), and *FRT-lacZ* fusion vectors (12, 37). The random *Himar1*-based mariner transposon plasmid pBT20 was successfully used for mutant library construction in *Pseudomonas aeruginosa* (6, 19, 22) and has also been proven useful for transposition in a broad range of gram-negative bacteria (20). Similarly, the *Himar1*-based transposons carrying the Km^r cassette were proven to be useful in *B. pseudomallei* (32). The second single-copy system based on the mini-Tn*7* site-specific transposon, when used in conjunction with the transposase-encoding helper plasmid, has broad applications for the introduction of single-copy chromosomal elements into gram-negative bacteria (9) and the select agent *B. mallei* (8). Lastly, after mutant construction with an *FRT*-flanked selectable marker and Flp excision, the introduction of an Flp-containing helper plasmid and an *FRT-lacZ* fusion vector allows for simple Flp-catalyzed recombination to the "*FRT* scar" at the target gene downstream of the native promoter, facilitating regulation studies without prior knowledge of the promoter sequence (12, 37). Nevertheless, there are disadvantages to these existing systems when used in *Burkholderia* species, particularly in the select agents *B. pseudomallei* and *B. mallei*, due to the antibiotic resistance markers used (e.g., gentamicin, kanamycin, ampicillin, and streptomycin) and the occurrence of spontaneously resistant mutants (10, 15, 32). Moreover, to our knowledge, no *FRT*-reporter fusion vectors based on reporter genes other than *lacZ* have been developed.

In this study, genetic tools using the Tel^r marker for selection were developed for single-copy analyses of chromosomally targeted genetic elements. These include a *Himar1*-based random mariner transposon plasmid and a mini-Tn*7* site-specific transposon vector. We also engineered *FRT*-reporter fusion vectors based on three common reporters, *lacZ*, *gfp*, and the *luxCDABE* operon, allowing for Flp-catalyzed recombination. These systems expand upon our previously published nonantibiotic selectable marker approach for allelic replacement (2) and will aid in routine genetic manipulations including transposon mutagenesis, complementation studies, and promoter regulation studies of *Burkholderia* species. Most importantly, all genetic tools presented here are completely devoid of antibiotic resistance selection and are in compliance with select-agent regulations. We utilized these tools to characterize the *B. thailandensis betBA* operon, encoding betaine aldehyde dehydrogenase (BetB) and choline dehydrogenase (BetA).

MATERIALS AND METHODS

Bacterial strains, media, and culturing conditions. All the strains and plasmids involved in this study are listed in Tables 1 and 2. *Escherichia coli* strain EPMax10B-*pir116* was routinely used as a cloning strain. *E. coli* strain DH5α-*pir* was used for the cloning of pBT20-Δ*bla*-Tel^r-*FRT*. *E. coli* strain E1345 was used to clone *E. coli asd* (asd_{Ec})-containing vectors. The *E. coli* conjugal and suicidal strain E1354 was routinely used for introducing plasmids into *Burkholderia* species through conjugation. An alternative *E. coli* conjugal donor, E463, was used for the conjugal transfer of transposon plasmid pBT20-Δ*bla*-Tel^r-*FRT*. Luria-Bertani (LB) medium (Difco) was used to culture all *E. coli*, *Burkholderia cenocepacia*, *B. pseudomallei*, and *B. mallei* strains. *B. thailandensis* wild-type strain E264 and its derivatives were cultured in LB medium or 1× M9 minimal medium supplemented with 20 mM glucose (MG). For the single-copy complementation study (see Fig. 3), *B. thailandensis* strains were grown in 1× M9 minimal medium plus 1% Brij 58 (Sigma) and 20 mM glucose or 30 mM choline chloride (Sigma). One percent Brij 58 was added to prevent bacterial clumping during growth. To study *betBA* regulation, *B. thailandensis* strains were grown in MG plus 1% Brij 58 along with different concentrations of choline chloride (see Fig. 5) or in no-salt LB medium (LS medium; Teknova) supplemented with different NaCl concentrations (see Fig. 6). Antibiotics and nonantibiotic bactericidal compounds were added to the media utilized for both selection and plasmid maintenance as follows: 110 μg/ml ampicillin (Ap), 25 μg/ml chloramphenicol (Cm), 15 μg/ml gentamicin (Gm), 35 μg/ml kanamycin (Km), 25 μg/ml, streptomycin (Sp), and 20 μg/ml potassium Tel (Teknova) for *E. coli*; 125 μg/ml Tel for *B. cenocepacia* strain K56-2 and *B. thailandensis*; 200 μg/ml Tel for *B. cenocepacia* strain J2315; and 25 μg/ml Tel for *B. pseudomallei* strains K96243 and 1026b and *B. mallei* strain ATCC 23344. For the growth of *E. coli* Δ*asd* strains E463, E1345, and E1354, without asd_{Ec}-containing plasmids, 100 μg/ml of diaminopimelic acid (Sigma) was supplied. All manipulations of *B. pseudomallei* and *B. mallei* were conducted in a CDC/USDA-approved and -registered biosafety level 3 facility at the University of Hawaii at Manoa. All experiments with these two select agents were performed with biosafety level 3 practices according to recommendations described previously (32a).

Molecular methods and reagents. All restriction enzymes, deoxynucleoside triphosphates, T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the supplier. Plasmids and DNA gel bands were isolated using the Zyppy plasmid miniprep kit I and Zymoclean gel DNA recovery kit, respectively (Zymo Research Corporation). Competent cells were prepared as previously described (2). All other molecular techniques were conducted according to methods described previously by Sambrook and Russell (33). Oligonucleotide primers (Table 3) were synthesized by Integrated DNA Technology. *Pfu* polymerase was purchased from Stratagene. Generally, the various PCRs were performed by an initial denaturation step for 1 min at 94°C and 34 cycles of 45 s at 94°C, 30 s at 60°C, and 1 min kb^{-1} at 72°C, and a final step for 10 min at 72°C was included.

Conjugal transfer of vectors into *Burkholderia* species. *E. coli* strain E463 was used as the conjugal donor to introduce transposon plasmid pBT20-Δ*bla*-Tel^r-*FRT* into all *Burkholderia* species. Another *E. coli* conjugal strain, E1354, was used to introduce the mini-Tn*7* and *FRT-lacZ* vectors and their respective helper plasmids into *B. thailandensis* strains. Conjugation of non-select-agent *Burkholderia* species was carried out as follows. The donor and recipients were grown to log phase for conjugation. One milliliter of each culture was harvested separately by centrifugation at 9,000 × *g* for 1 min at room temperature and washed twice with 1 ml of LB medium. The cell pellets of the donor and recipients were then resuspended together in 30 μl of LB medium. The 30-μl cell suspension was spotted onto cellulose acetate filters (Satorius) on LB agar plates and incubated at 37°C for 8 h. Filters were then vortexed in 1 ml of 1× M9 minimal medium, and 100 μl of this cell suspension and 100 μl of 10× dilutions were plated onto LB or MG plates with appropriate concentrations of Tel. Conjugations into *B. pseudomallei* or *B. mallei* cells were performed directly on LB plates without filters. Bacteria were gently scraped off the LB plates with disposable inoculation loops and resuspended in 1× M9 medium, and plating was done similarly as described above. Plates were usually incubated for 2 to 3 days at 37°C until single Tel^r colonies were observed.

TABLE 1. Bacterial strains utilized in this study

Strain	Lab ID[a]	Relevant characteristic(s)	Source or reference
E. coli			
EPMax10B-*pir116*	E1249	F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL nupG* Tn-*pir116-FRT2*	Laboratory collection
DH5α-*pir*	E0175	Tc^r; F$^-$ λ$^-$ ϕ80d*lacZ*ΔM15 (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1 uidA::pir zdg-232*::Tn*10*	31
HPS1-*mob*-Δ*asd-pir*	E0463	Tc^r Km^r Cm^r; e14$^-$ (*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*) *rif zxx*::mini-Tn*5*-Lac4 (*lacI*$^{q+}$ *lacZ* M15) Δ*asd*::*FRT uidA*::*pir zdg-232*::Tn*10 recA*::*RP4-2* Tc::Mu Km^r	—[b]
EPMax10B-*pir116*-Δ*asd*::Gm^r	E1345	Gm^r; F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL nupG* Tn-*pir116-FRT2* Δ*asd*::Gm^r	—[b]
EPMax10B-*pir116*-Δ*asd-mob*-Km^r-Δ*trp*::Gm^r	E1354	Km^r Gm^r; F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL nupG* Tn-*pir116-FRT2* Δ*asd*::*FRT recA*::*RP4-2* Tc::Mu Km^r Δ*trp*::Gm^r	—[b]
DH5α-λ*attB*::pCD13SK-Flp	E0982	Sp^r; F$^-$ ϕ80d*lacZ*ΔM15 (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ*attB*::pCD13SK-Flp	—[b]
B. thailandensis			
E264	E1298	Prototroph	5
E264-Δ*betBA*::*FRT*	E1671	*B. thailandensis* Δ*betBA*::*FRT* mutant	2
E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r	E1709	Tel^r; *B. thailandensis* Δ*betBA*::*FRT* mutant with empty vector mini-Tn*7*-Tel^r integrated at the *att*Tn*7* site	This study
E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA*	E1711	Tel^r; *B. thailandensis* Δ*betBA*::*FRT* mutant with mini-Tn*7*-Tel^r-*betBA* integrated at the *att*Tn*7* site	This study
E264-Δ*betBA*::*FRT-lacZ*-Tel^r	E1731	Tel^r; *B. thailandensis* Δ*betBA*::*FRT* mutant with *FRT-lacZ*-Tel^r fusion	This study
E264-Δ*betBA*::*FRT-gfp*-Tel^r	E2045	Tel^r; *B. thailandensis* Δ*betBA*::*FRT* mutant with *FRT-gfp*-Tel^r fusion	This study
E264-Δ*betBA*::*FRT-lux*-Tel^r	E2047	Tel^r; *B. thailandensis* Δ*betBA*::*FRT* mutant with *FRT-lux*-Tel^r fusion	This study
E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*	E1849	Tel^r; E264-Δ*betBA*::*FRT*/*attTn7*::*betBA* with Δ*betBA-lacZ*-Tel^r fusion	This study
E264-Δ*betBA*::*FRT-gfp*-Tel^r/*attTn7*::*betBA*	E2046	Tel^r; E264-Δ*betBA*::*FRT*/*attTn7*::*betBA* with Δ*betBA-gfp*-Tel^r fusion	This study
E264-Δ*betBA*::*FRT-lux*-Tel^r/*attTn7*::*betBA*	E2048	Tel^r; E264-Δ*betBA*::*FRT*/*attTn7*::*betBA* with Δ*betBA-lux*-Tel^r fusion	This study
B. cenocepacia			
K56-2	E1554	Prototroph; cystic fibrosis isolate	P. Sokol
J2315	E1553	Prototroph	J. Goldberg
B. pseudomallei			
K96243	B0005	Prototroph; clinical isolate	18
1026b	B0003	Prototroph; clinical isolate	11
B. mallei ATCC 23344	B0001	Prototroph; clinical isolate	40

[a] For strains constructed in this study, please see the text for further details. Please use the laboratory identification (Lab ID) number to request strains.
[b] —, details on the engineering of these strains will be published elsewhere.

Construction and testing of pBT20-Δ*bla*-Tel^r-*FRT*. A new mariner transposon vector was constructed based on the Tel^r marker (Fig. 1). pwFRT-PC$_{S12}$-Tel^r was digested with SmaI, and the 3.2-kb PC$_{S12}$-Tel^r fragment was cloned into the pBT20-Δ*bla* backbone (~4.3 kb) following BsaI digestion and blunt ending. This replaced the Gm^r cassette with the Tel^r marker, resulting in transposon vector pBT20-Δ*bla*-Tel^r-*FRT*.

The transposition frequencies of pBT20-Δ*bla*-Tel^r-*FRT* were determined by conjugation into several different *Burkholderia* species and strains: *B. cenocepacia* strains K56-2 and J2315, *B. thailandensis* strain E264, *B. pseudomallei* strains K96243 and 1026b, and *B. mallei* strain ATCC 23344. Following conjugation as described above, the mating mixtures were diluted and plated onto LB plates and LB plates supplemented with the appropriate concentration of Tel. The transposition frequencies for individual conjugation experiments were calculated based on the ratio of the number of colonies counted that were grown on LB medium plus Tel to the number of colonies that were grown on LB medium. Three independent conjugation experiments were carried out to obtain the average transposition frequency and standard error of the mean for each strain. Similar control conjugation experiments, omitting the *E. coli* conjugal donor harboring pBT20-Δ*bla*-Tel^r-*FRT*, were performed on all recipient strains to ensure that no spontaneous mutants arose from Tel selection. For *B. thailandensis* and *B. cenocepacia*, 15 random Tel^r colonies were purified on LB plates with Tel and PCR screened using *telB*-specific oligonucleotides 834 and 854

TABLE 2. Bacterial plasmids utilized in this study

Plasmid	Lab ID[a]	Relevant properties	Reference or source
pBT20-Δ*bla*	E1029	Gm^r; mariner transposon plasmid	20
pwFRT-P$_{S12}$-Tel^r	E1584	Tel^r; P$_{S12}$-Tel^r cassette flanked by wild-type *FRT* sequences	2
pBT20-Δ*bla*-Tel^r-*FRT*	E1727	Tel^r; mariner transposon plasmid based on Tel^r	This study
pCD11-Gm^r-*pir116-oriT*	E1254	Cm^r Gm^r; conjugation vector	Laboratory collection
pUC18R6KT-mini-Tn*7*	E1190	Ap^r; Tn*7*-based broad-host-range transposon vector	9
mini-Tn*7*-Tel^r-*bla*	E1645	Ap^r Tel^r; Tel^r cassette cloned into pUC18R6K-mini-Tn*7*	This study
mini-Tn*7*-Tel^r	E1825	Tel^r; mini-Tn*7*-*bla*-Tel^r with *bla* gene deleted	This study
mini-Tn*7*-Tel^r-*betBA*	E1829	Tel^r; mini-Tn*7*-Tel^r with *betBA* operon cloned	This study
pTNS3	E1189	Ap^r; helper plasmid for Tn*7* transposition system	9
pTNS3-*asd*$_{Ec}$	E1831	pTNS3 with *bla* replaced by the *E. coli asd* gene	This study
pFRT1-*lacZ*	E0790	Gm^r; *FRT1-lacZ* fusion containing suicidal vector	37
pFRT1-*lacZ*-Tel^r	E1707	Tel^r; pFRT1-*lacZ* with Gm^r cassette replaced by Tel^r cassette	This study
pFRT2-*lacZ*	E0787	Gm^r; *FRT2-lacZ* fusion containing suicidal vector	37
pFRT2-*lacZ*-Tel^r	E1708	Tel^r; pFRT2-*lacZ* with Gm^r cassette replaced by Tel^r cassette	This study
pPS856-ΔXbas	E1044	Gm^r Ap^r; Gm^r cassette flanked by wild-type *FRT* sequences	—[b]
pPS747	E0042	Ap^r; *gfp*-containing vector	17
pAKlux2	E1863	Ap^r; *luxCDABE* bioluminescence operon-containing vector	21
pFRT1-Gm^r-*lacZ*-Tel^r	E2049	Gm^r Tel^r; pFRT1-*lacZ*-Tel^r with *FRT1* replaced by the *FRT1*-Gm^r-*FRT1* fragment	This study
pFRT2-Gm^r-*lacZ*-Tel^r	E2050	Gm^r Tel^r; pFRT1-*lacZ*-Tel^r with *FRT1* replaced by the *FRT2*-Gm^r-*FRT2* fragment	This study
pFRT1-*lacZ*-Tel^r-ΔBam	E2051	Tel^r; pFRT1-Gm^r-*lacZ*-Tel^r with Flp-excised *FRT1*-Gm^r	This study
pFRT2-*lacZ*-Tel^r-ΔBam	E2052	Tel^r; pFRT2-Gm^r-*lacZ*-Tel^r with Flp-excised *FRT2*-Gm^r	This study
pFRT1-*gfp*-Tel^r	E2053	Tel^r; pFRT1-*lacZ*-Tel^r-ΔBam with *gfp* replacing *lacZ*	This study
pFRT2-*gfp*-Tel^r	E2055	Tel^r; pFRT2-*lacZ*-Tel^r-ΔBam with *gfp* replacing *lacZ*	This study
pFRT1-*lux*-Tel^r	E2064	Tel^r; pFRT1-*lacZ*-Tel^r-ΔBam with *luxCDABE* replacing *lacZ*	This study
pFRT2-*lux*-Tel^r	E2066	Tel^r; pFRT2-*lacZ*-Tel^r-ΔBam with *luxCDABE* replacing *lacZ*	This study
pCD13SK-Flp-*oriT*	E0803	Sp^r; Flp-containing suicidal vector	37
pCD13SK-Flp-*oriT*-*asd*$_{Ec}$	E1827	pCD13SK-Flp-*oriT* with *asd*$_{Ec}$ replaced by the Sp^r cassette	This study
pFlpAB-5	E1662	Tp^r; broad-host-range Flp-containing vector	2

[a] For plasmids constructed in this study, please see the text for further details. Please use the laboratory identification (Lab ID) number when requesting plasmids.
[b] —, details on the engineering of this plasmid are to be published elsewhere.

(Table 3). For *B. pseudomallei* and *B. mallei*, five random Tel^r colonies were purified on LB plates with Tel and PCR screened using *kilA* oligonucleotides 831 and 1066, *telA* oligonucleotides 827 and 1067, and *telB* oligonucleotides 834 and 854 (Table 3). Southern hybridization analysis was also performed, as described previously (17), for the *B. pseudomallei* and *B. mallei* Tel^r colonies using a *telB*-specific probe after the digestion of chromosomal DNA with XhoI.

Construction of mini-Tn*7* site-specific transposon vectors and their helper plasmid. The mini-Tn*7*-Tel^r site-specific transposon, based on Tel^r, was constructed as described below. pwFRT-PC$_{S12}$-Tel^r was digested with SacI and blunt ended, and the PC$_{S12}$-Tel^r fragment (3.2 kb) was cloned into pUC18R6KT-mini-Tn*7* (EcoRV digested), resulting in mini-Tn*7*-Tel^r-*bla*. The R6K*γori*-*oriT* region, amplified from pCD11-Gm^r-*pir116-oriT* using oligonucleotides 696 and 702, was ligated with the mini-Tn*7*-Tel^r-*bla* backbone (including the Tel^r cassette) following AflII and NarI digestion and blunt ending. This resulted in a mini-Tn*7*-Tel^r vector (Fig. 2A).

TABLE 3. Oligonucleotide primers utilized in this study

Primer (name)	Sequence[a]
89 (*asd*$_{Ec}$-up)	5′-CGGTTGAATTCTACTCCGGTGCGCAAATGGC-3′
91 (*asd*$_{Ec}$-down)	5′-TACTGAATTCCGCCAAAATGGCCTGCAATTA-3′
696 (*oriT*-ClaI-1)	5′-TGGGTATCGATTCCTTAAGGTATACTTT-3′
702 (*R6K*)	5′-TGTCAGCCGTTAAGTGTTCC-3′
713 (*lacZ*α)	5′-TGTTGGGAAGGGCGATC-3′
827 (*telA*-SmaI)	5′-GGGAACGACCCTGGCCGCGTGCA-3′
831 (*tel-kilA*)	5′-AGCTAAAATGGAAGAACAAA-3′
834 (*telB*-XhoI)	5′-CCTCCTCGAGCAGAAAGTCAAAAGCCTC-3′
854 (*telB*-down)	5′-TACCAGCAGGAATGGAAC-3′
861 (Bt-*betBA*-HindIII)	5′-CCCGCAAGCTTGCCGGCAA-3′
862 (Bt-*betBA*-KpnI)	5′-GACCGGTACCCGGCGGGCGGGGATAT-3′
874 (*glmS1*-DN)[b]	5′-GTTCGTCGTCCACTGGGATCA-3′
875 (*glmS2*-DN)[b]	5′-AGATCGGATGGAATTCGTGGAG-3′
876 (Tn*7*L)[b]	5′-ATTAGCTTACGACGCTACACCC-3′
885 (*gfp*-BspHI-down)	5′-CAGGTCATGACACCTCTCTTTATTTGTATAGTTC-3′
1030 (*lux*-rev)	5′-GGATTGCACTAAATCATC-3′
1066 (*kilA*-rev)	5′-TCGGCTTCGTCCAGCAAC-3′
1067 (*telA*-rev)	5′-GCATTGCGCTTCATCAGG-3′

[a] Restriction enzyme sites utilized in this study are underlined.
[b] Oligonucleotides were synthesized as previously described (9).

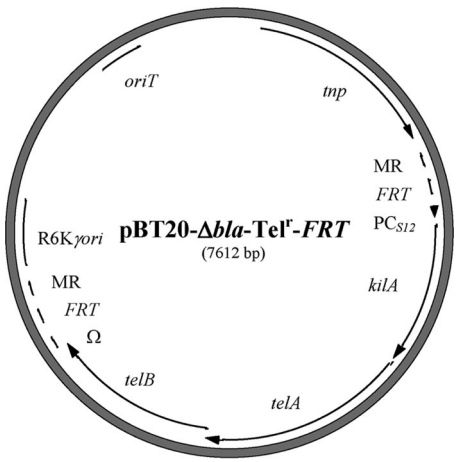

FIG. 1. Plasmid map of mariner transposon vector pBT20-Δ*bla*-Tel^r-*FRT* based on Tel^r. The Tel^r cassette, consisting of the *kilA*, *telA*, and *telB* genes, is flanked by two identical *FRT* sequences. A *B. cenocepacia* PC$_{S12}$ promoter was included upstream of the Tel^r cassette. Abbreviations: Ω, *tonB* transcriptional terminator; *FRT*, Flip recombination target; *oriT*, conjugal origin of transfer; MR, mariner repeats; PC$_{S12}$, *B. cenocepacia rpsL* promoter; R6K*γori*, suicidal R6K origin of replication; *tnp*, mariner transposase gene.

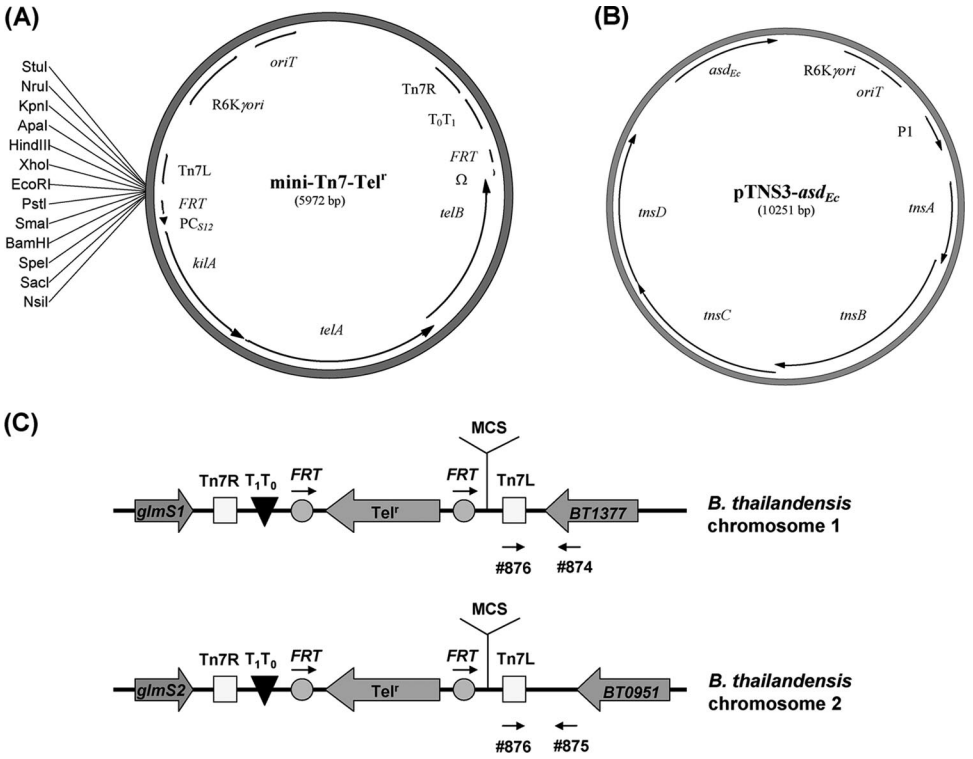

FIG. 2. The mini-Tn7-Tel^r integration vector and its pTNS3-*asd*$_{Ec}$ helper plasmid. (A) mini-Tn7-Tel^r, a Tn7-based suicidal vector with the Tel^r marker. (B) pTNS3-*asd*$_{Ec}$ suicidal helper plasmid encoding the transposase, which catalyzes Tn*7* transposition. (C) Chromosomally inserted mini-Tn7-Tel^r elements at two different *B. thailandensis att*Tn*7* sites as previously described (6). Oligonucleotides 874, 875, and 876, indicated by arrows, were used to screen for the location of transposition. *glmS1* and *glmS2* encode glucosamine-6-phosphate synthetases; MCS, multiple-cloning site; P1, P1 integron promoter; Tn7L, Tn*7* transposase left recognition sequence; Tn7R, Tn*7* transposase right recognition sequence; T_0T_1, transcriptional terminators; *tnsABCD*, Tn*7* transposases.

Helper plasmid pTNS3-*asd*$_{Ec}$ (Fig. 2B), containing the Tn*7* transposase genes, was also constructed based on *asd*$_{Ec}$. The *asd*$_{Ec}$ fragment was amplified from *E. coli* K-12 chromosomal DNA using oligonucleotides 89 and 91. The 2.5-kb PCR product was digested with EcoRI and PstI and blunt ended, and the 1.6-kb *asd*$_{Ec}$ gene was ligated into the pTNS3 backbone (digested with BglI and blunt ended), yielding pTNS3-*asd*$_{Ec}$.

Single-copy complementation of E264-Δ*betBA*::*FRT*. The mini-Tn7-Tel^r and the *betBA* operon fragment (amplified from E264 with oligonucleotides 861 and 862) were digested with HindIII and KpnI and ligated together, yielding mini-Tn7-Tel^r-*betBA*. The *B. thailandensis* E264-Δ*betBA*::*FRT* mutant was complemented using the constructed mini-Tn7-Tel^r-*betBA*. Two E1354 strains, each harboring either mini-Tn7-Tel^r-*betBA* or helper plasmid pTNS3-*asd*$_{Ec}$, were used to perform triparental matings with the *B. thailandensis* recipient E264-Δ*betBA*::*FRT*, creating the E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA* complemented strain. As a control, an empty mini-Tn7-Tel^r vector was conjugated into the E264-Δ*betBA*::*FRT* strain, resulting in E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r. *B. thailandensis* transconjugants with the mini-Tn7 transposon inserted at the *att*Tn7 site were selected on LB plates with Tel and screened by PCR using oligonucleotides 874 and 876 or oligonucleotides 875 and 876 (Fig. 2C).

Characterization of growth of E264-Δ*betBA*::*FRT* and the complemented strain. Growth curve experiments were performed on three *B. thailandensis* strains: wild-type E264, the E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r control, and the E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA* complement. These strains were grown overnight at 37°C in LB medium. Cultures grown overnight were washed twice with 1 volume of 1× M9 buffer and resuspended in an equal volume of the same buffer. Resuspended cultures were then diluted 100-fold into a solution containing fresh 1× M9 medium, 1% Brij 58, and 20 mM glucose or 30 mM choline chloride, and growth was initiated by shaking at 250 rpm and 37°C. At all time points, aliquots of each culture were taken, and optical densities were measured at 600 nm.

Construction of *FRT-lacZ*, *FRT-gfp*, and *FRT-lux* fusion vectors. An *flp*-carrying helper plasmid containing a nonantibiotic resistance marker was first created for the recombination of the various reporter fusion vectors. pCD13SK-Flp-*oriT* was digested with NdeI and SalI, blunt ended, and ligated with the above-mentioned 1.6-kb *asd*$_{Ec}$ fragment. The resulting helper plasmid, pCD13SK-Flp-*oriT*-*asd*$_{Ec}$ (see Fig. 4A), contains the *asd*$_{Ec}$ marker in place of the Sp^r cassette.

Two pFRT-*lacZ*-Tel^r vectors, pFRT1-*lacZ*-Tel^r and pFRT2-*lacZ*-Tel^r (Fig. 4B), were constructed in this study by replacing the Gm^r cassette with the Tel^r cassette. To create pFRT1-*lacZ*-Tel^r, pwFRT-PC$_{S12}$-Tel^r was digested with EcoRV and XhoI and blunt ended, and the resulting PC$_{S12}$-Tel^r fragment was cloned into pFRT1-*lacZ* (digested with BsrGI and SacII and blunt ended to remove the Gm^r cassette). Similarly, pFRT2-*lacZ*-Tel^r was constructed by cloning the PC$_{S12}$-Tel^r fragment into the pFRT2-*lacZ* backbone.

Four different fusion vectors were constructed based on the *gfp* reporter gene and the *luxCDABE* operon. In order to replace the *lacZ* gene with the promoterless *gfp* reporter gene or the *lux* operon, several cloning steps were carried out to eliminate one of the BamHI sites flanking the *FRT* sequence, leaving a unique BamHI site downstream of the *FRT* sequence. First, pPS856-ΔXbas was digested with SmaI to recover the *FRT*-Gm^r-*FRT* fragment, which was cloned into the pFRT1-*lacZ*-Tel^r backbone following digestion with the same enzyme. This cloning step resulted in the creation of pFRT1-Gm^r-*lacZ*-Tel^r (with the Gm^r cassette in the same orientation as the *lacZ* gene) and pFRT2-Gm^r-*lacZ*-Tel^r (with the Gm^r cassette in the opposite orientation of the *lacZ* gene). To Flp excise the Gm^r-*FRT* fragment, both pFRT1-Gm^r-*lacZ*-Tel^r and pFRT2-Gm^r-*lacZ*-Tel^r were introduced into Flp-containing strain DH5α-λ*attB*::pCD13SK-Flp. The resulting constructs, pFRT1-*lacZ*-Tel^r-ΔBam and pFRT2-*lacZ*-Tel^r-ΔBam, were digested with NdeI, blunt ended, and then digested with BamHI. These Tel^r cassette-containing vectors were ligated with the *gfp* gene from pPS747 (digested with HindIII, blunt ended, and then digested with BamHI), yielding pFRT1-*gfp*-Tel^r and pFRT2-*gfp*-Tel^r, respectively (Fig. 4C). Finally, pFRT1-*lacZ*-Tel^r-ΔBam and pFRT2-*lacZ*-Tel^r-ΔBam were digested with BamHI and NdeI and blunt ended, and the *luxCDABE* operon, obtained from pAKlux2 (Addgene plasmid 14080) following EcoRI digestion and blunt ending, was cloned to yield pFRT1-*lux*-Tel^r and pFRT2-*lux*-Tel^r (Fig. 4D).

Engineering of *B. thailandensis* E264-Δ*betBA*::*FRT* reporter fusion strains. To construct the E264-Δ*betBA*::*FRT-lacZ*-Tel^r reporter strain for the *betBA* promoter study, pFRT1-*lacZ*-Tel^r and the helper plasmid (pCD13SK-Flp-*oriT*-*asd*$_{Ec}$) were conjugated from E1354 into E264-Δ*betBA*::*FRT* in a triparental mating experiment. Colonies on MG plates with 125 μg/ml Tel were screened by PCR using oligonucleotides 713 and 861 to confirm the correct orientation of the *lacZ* gene relative to the *betBA* promoter region. This strain was then used in the choline induction study (see Fig. 5B and C).

The E264-Δ*betBA*::*FRT-lacZ*-Tel^r fusion strain was complemented by engineering the E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA* strain in several steps (see Fig. 6A). First, the Tel^r cassette in strain E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA* (described above) was Flp excised using pFLP-AB5 according to a previously described procedure (2). The resulting strain, E264-Δ*betBA*::*FRT*/*attTn7*::*betBA*, was conjugated with two E1354 donor strains harboring either pFRT1-*lacZ*-Tel^r or pCD13SK-Flp-*oriT*-*asd*$_{Ec}$ in a triparental mating experiment. This triparental mating mixture was then plated onto MG plates with 125 μg/ml Tel to select for fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*, harboring a *lacZ* reporter driven by the native *betBA* promoter. Colonies were screened by PCR using oligonucleotides 713 and 861 (Fig. 6A). Isolates with *lacZ* integrated at the *betBA* locus were purified once on LB medium and used in the osmotic regulation study (see Fig. 6B and C).

Fusion vector pFRT1-*gfp*-Tel^r was also integrated into the Δ*betBA*::*FRT* mutant and complemented strains as described above. The resulting fusion strains, E264-Δ*betBA*::*FRT-gfp*-Tel^r and E264-Δ*betBA*::*FRT-gfp*-Tel^r/*attTn7*::*betBA*, were screened by PCR using oligonucleotides 861 and 885 (Fig. 6A). Similarly, vector pFRT1-*lux*-Tel^r was used to construct fusion strains E264-Δ*betBA*::*FRT-lux*-Tel^r and E264-Δ*betBA*::*FRT-lux*-Tel^r/*attTn7*::*betBA*, which were screened by PCR using oligonucleotides 861 and 1030 (see Fig. 6A).

Choline and osmotic regulation studies of the *betBA* operon. β-Galactosidase activity of the integrated *betBA*::*FRT-lacZ*-Tel^r fusion was measured under various growth conditions. To study choline induction of the *betBA* operon, fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r was grown overnight in LB medium. Cultures grown overnight were washed twice with 1 volume of 1× M9 medium and resuspended in an equal volume of the same medium. Resuspended cultures were then diluted 100-fold into a solution containing fresh 1× M9 medium, 1% Brij 58, 20 mM glucose, and 0, 1, 2, 4, or 8 mM choline chloride. Growth curve experiments were performed on each culture by diluting sample aliquots twice in 4% Brij 58 and measuring the optical density at 600 nm (see Fig. 5B). Additional 1-ml cell culture aliquots were taken at each time point during the growth curve experiments to assay for β-galactosidase activity. These assays were done in triplicate and are displayed as average Miller units (28), with standard errors of the means (see Fig. 5C). *gfp* fusion strain E264-Δ*betBA*::*FRT-gfp*-Tel^r was grown in a solution containing 1× M9 medium, 1% Brij 58, and 20 mM glucose with or without 8 mM choline chloride to early stationary phase (~36 h), at which point wet mounts were prepared and examined under an Olympus BX51 fluorescent microscope to assay fluorescent activity in the presence or absence of choline (Fig. 5D). Fusion strain E264-Δ*betBA*::*FRT-lux*-Tel^r was grown in 1× M9 medium with 1% Brij 58 and 20 mM glucose with or without 8 mM choline chloride to early stationary phase, at which point 1 ml of each culture was centrifuged, and the cell pellet was resuspended with 20 μl of 1× M9 medium. The resuspended cells were then spotted onto an MG plate, and images were obtained immediately using a Bio-Rad biochemiluminescent imaging system.

To study the NaCl-mediated osmotic regulation of the *betBA* operon, we complemented the Δ*betBA* mutant because NaCl significantly affected the growth in the absence of the *betBA* operon. Growth curve experiments were conducted on the complemented fusion strain (E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*) and the wild-type strain (E264) by first growing cultures overnight in LS medium. Cultures grown overnight were washed with 1 volume of LS medium, resuspended in an equal volume of the same medium, and diluted 100-fold into fresh LS medium with 0, 0.3, and 0.4 M NaCl. Growth curve experiments were performed for each culture by taking optical density measurements at 600 nm (see Fig. 6B). Additional 1-ml cell culture aliquots of E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA* were taken at each time point during the growth curve experiments to assay for β-galactosidase activity (Fig. 6C). Two other fusion strains, E264-Δ*betBA*::*FRT-gfp*-Tel^r/*attTn7*::*betBA* and E264-Δ*betBA*::*FRT-lux*-Tel^r/*attTn7*::*betBA*, were grown in LS medium with or without 0.3 M NaCl to late log phase (~30 h), at which time fluorescent microscopy and bioluminescence imaging analyses (see Fig. 6D and E) were performed as described above.

Nucleotide sequence accession numbers. All sequences of vectors presented in Fig. 1, 2, and 4 were submitted to the GenBank database. The accession numbers are as follows: EU626135 for pBT20-Δ*bla*-Tel^r-*FRT*, EU626136 for mini-Tn*7*-Tel^r, FJ797680 for pTNS3-*asd*$_{Ec}$, EU626138 for pCD13SK-Flp-*oriT*-*asd*$_{Ec}$, EU626139 for pFRT1-*lacZ*-Tel^r, EU626140 for pFRT2-*lacZ*-Tel^r, FJ455408 for pFRT1-*gfp*-Tel^r, FJ455409 for pFRT2-*gfp*-Tel^r, FJ455410 for pFRT1-*lux*-Tel^r, and FJ455411 for pFRT2-*lux*-Tel^r.

TABLE 4. Transposition frequencies of pBT20-Δ*bla*-Tel^r-*FRT* in *Burkholderia* species

Species (strain)	Avg frequency of transposition ± SEM[a]
B. cenocepacia (K56-2)	$(1.22 \pm 0.21) \times 10^{-5}$
B. cenocepacia (J2315)	$(2.34 \pm 0.33) \times 10^{-6}$
B. thailandensis (E264)	$(4.29 \pm 0.42) \times 10^{-6}$
B. pseudomallei (K96243)	$(1.51 \pm 0.23) \times 10^{-5}$
B. pseudomallei (1026b)	$(1.07 \pm 0.10) \times 10^{-5}$
B. mallei (ATCC 23344)	$(2.08 \pm 0.16) \times 10^{-6}$

[a] All experiments were performed in triplicate, and averages are shown with the standard error of the mean.

RESULTS

Engineering and utilization of a random Tel^r transposon in *Burkholderia* species. A *Himar1*-based mariner transposon carrying a Km^r marker has been used successfully in *B. pseudomallei* although with some reported leakiness (32). To further develop and test a mariner transposon based on the alternative nonantibiotic Tel^r marker for a broader range of *Burkholderia* spp., we replaced the Gm^r marker on mariner transposon plasmid pBT20. pBT20, originally based on the Gm^r marker with a *bla* gene in its plasmid backbone, is not appropriate for selection in *Burkholderia* species due to their high level of Gm^r and may be inappropriate for use in the select-agent species *B. pseudomallei* and *B. mallei*. In this study, we constructed a mariner transposon, pBT20-Δ*bla*-Tel^r-*FRT*, based on the nonantibiotic Tel^r marker (Fig. 1) previously shown to be effective in *B. thailandensis* (2). We eliminated the *bla* gene from pBT20 and replaced the Gm^r cassette on the transposon with the Tel^r marker for selection in both *E. coli* and *Burkholderia*. To demonstrate the effectiveness of this transposon, we conjugated pBT20-Δ*bla*-Tel^r-*FRT* from a suicidal Δ*asd E. coli* strain into four different *Burkholderia* species: *B. cenocepacia* (two strains), *B. thailandensis*, *B. pseudomallei* (two strains), and *B. mallei*. For each species, three independent mating experiments were conducted, and the average transposition frequencies were determined and are shown in Table 4. We determined the effective Tel concentrations for the four *Burkholderia* species, and no spontaneous Tel^r mutants were detected when 10^9 CFU were plated alone on LB medium with Tel as controls (see Materials and Methods). On average, conjugation mixtures were resuspended in 1 ml of LB medium and diluted 10×, where 100-μl volumes were plated onto LB medium with Tel, yielding 50 to 200 colonies depending on the species. Fifteen random Tel^r colonies from *B. cenocepacia* (J2315 and K56-2) and *B. thailandensis* were screened by PCR using *telB*-specific oligonucleotides, and five random colonies from *B. pseudomallei* (K96243 and 1026b) and *B. mallei* were positively screened by PCR using *kilA*-, *telA*-, and *telB*-specific oligonucleotides (see Fig. S1A to S1F in the supplemental material). Southern blot analysis was also performed on the 15 Tel^r isolates of *B. pseudomallei* and *B. mallei* using a *telB*-specific probe (see Fig. S1G in the supplemental material). Single bands with different sizes were obtained in all isolates, suggesting random transposition into the *B. pseudomallei* and *B. mallei*

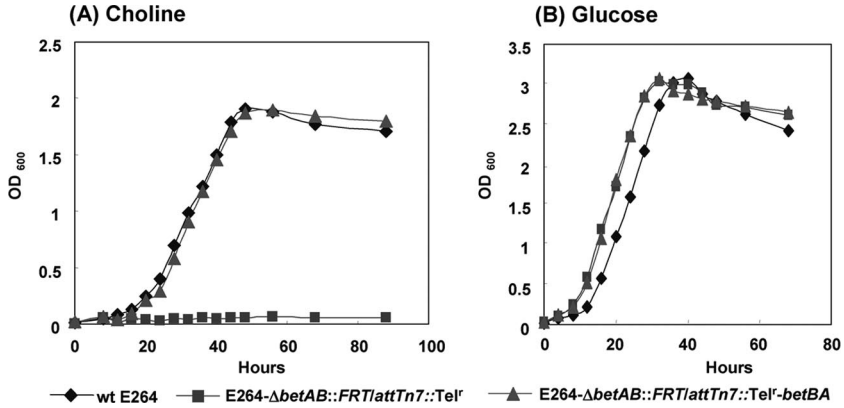

FIG. 3. Growth analyses of the *B. thailandensis* Δ*betBA* mutant and its complement on choline and glucose. Wild-type strain E264, the E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r mutant, and the E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA* complement were grown in 1× M9 minimal medium supplemented with 30 mM choline (A) or 20 mM glucose (B). All three strains exhibited similar growth rates and overall cell densities when grown in glucose. The complemented strain, E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA*, displayed the same growth rate as the wild-type (wt) strain on choline. However, the *betBA* mutant strain containing the empty Tn*7* transposon control, E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r, was not able to grow on choline as a sole carbon source. OD_{600}, optical density at 600 nm.

genomes (see Fig. S1G in the supplemental material). All *Burkholderia* species tested displayed similar transposition frequencies, ranging from $2.08 \times 10^{-6} \pm 0.16 \times 10^{-6}$ to $1.51 \times 10^{-5} \pm 0.23 \times 10^{-5}$ (Table 4), which are comparable to the frequencies obtained when using a Km^r-based transposon in *B. pseudomallei* (32). However, analyses of 5 to 15 Tel^r colonies showed that 100% of the Tel^r colonies contained the transposon, demonstrating the effectiveness of Tel^r selection (see Fig. S1 in the supplemental material). No spontaneous resistance was observed when 10^9 CFU were plated, indicating that the spontaneous resistance frequency is $<10^{-9}$. If required, the transposon insertion sites could easily be determined by sequencing the flanking region of the transposon using semirandom PCR methods as previously described (19, 24). The Tel^r cassette in our transposon, flanked by *FRT* sequences (17), could then be excised by Flp recombinase for subsequent recycling of the Tel^r cassette or integration of *FRT*-reporter fusions (below) at the transposed loci for immediate gene regulation studies.

Engineering and testing of the single-copy mini-Tn*7*-Tel^r site-specific transposon by complementing the *B. thailandensis* Δ*betBA* mutant. The mini-Tn*7* site-specific transposon and helper plasmid (carrying the Tn*7* transposase genes) were utilized in various species (1, 3, 8, 23). This system could be used for single-copy complementation studies, promoter-reporter fusion integration, and reporter gene (e.g., fluorescence and bioluminescence proteins) tagging in *Burkholderia* species (1, 3, 8, 23). However, all these systems contain antibiotic resistance markers for selection, requiring the need for reengineering with nonantibiotic selectable markers. In this study, we developed a mini-Tn*7*-Tel^r site-specific transposon and a helper plasmid for *Burkholderia* species based on two nonantibiotic selectable markers, the Tel^r cassette and the asd_{Ec} gene, respectively (Fig. 2A and B). This mini-Tn*7*-Tel^r vector contains a multiple-cloning site for conveniently cloning genes of interest for subsequent site-specific transposition, which is catalyzed by the transposase encoded on the pTNS3-asd_{Ec} helper plasmid. The location of the chromosomally inserted transposon could be determined by PCR with site-specific and transposon-specific oligonucleotides (9) (Fig. 2C). The *FRT*-flanked Tel^r cassette allows Flp-catalyzed excision of the Tel^r marker while maintaining the introduced gene of interest at the specific transposition site.

As a proof of concept, the mini-Tn*7*-Tel^r system was used to complement the *betBA* mutation in *B. thailandensis*. Previously, we engineered a *B. thailandensis* Δ*betBA* mutant that exhibits a growth defect when grown in choline as a sole carbon source (2). A wild-type copy of the *betBA* operon was cloned into the multiple-cloning site of mini-Tn*7*-Tel^r (Fig. 2A), resulting in the mini-Tn*7*-Tel^r-*betBA* vector. The helper plasmid pTNS3-asd_{Ec} and the mini-Tn*7*Tel^r-*betBA* vector were simultaneously conjugated by triparental mating into strain E264-Δ*betBA*::*FRT*. Site-specific transposition of the *betBA*-Tel^r complement was confirmed by PCR as previously described (9). In the majority of Tel^r isolates (8 out of 10 screened), the mini-Tn*7* transposon was inserted downstream of the *glmS2* gene on the second chromosome, while two transpositions occurred downstream of the *glmS1* gene on the first chromosome (9). None of the isolates displayed integration on both chromosomes (see Fig. S2 in the supplemental material).

To show the complementation of the Δ*betBA* mutant, the constructed strain E264-Δ*betBA*::*FRT*/*attTn7*::Tel^r-*betBA* was tested for its ability to grow on choline as a sole carbon source. As shown in Fig. 3A, this chromosomally integrated copy of the *betBA* operon recovered the growth ability of the Δ*betBA* mutant strain on choline as a sole carbon source, displaying a growth rate and an overall cell density comparable to those of wild-type strain E264. The transposition of the empty mini-Tn*7*-Tel^r control into the Δ*betBA* mutant yielded no complementation of the Δ*betBA* mutation, and it was unable to grow with choline as a sole carbon source (Fig. 3A). Growth curve studies for these three strains on glucose as a sole carbon source were also conducted (Fig. 3B) to show that the integrated mini-Tn*7* system did not alter any other growth phenotypes of mutant strain E264-Δ*betBA*::*FRT*.

Engineering of reporter gene constructs and regulation studies of the *betBA* operon. Recombination of the *FRT-lacZ* reporter fusion with the single chromosomally located "*FRT* scar"

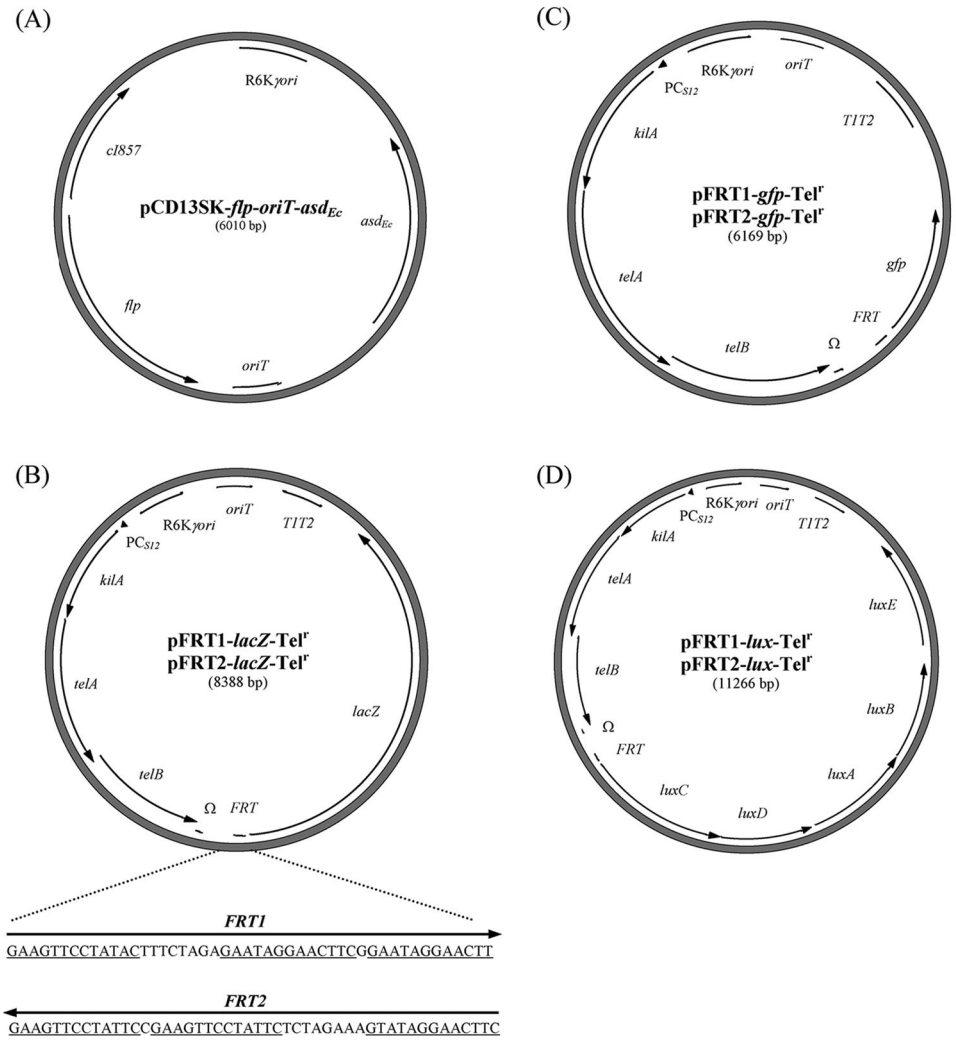

FIG. 4. Plasmid maps of the *FRT*-reporter fusion vectors and their helper plasmids. (A) pCD13SK-Flp-*oriT-asd*$_{Ec}$ is a *flp*-containing helper plasmid for the recombination of all *FRT*-reporter fusions. (B to D) Various fusion vectors were constructed based on the reporters *lacZ* (B), *gfp* (C), and the *luxCDABE* operon (D). With the exception of the *FRT* oriented relative to the reporter genes, all of the paired pFRT1-Tel^r and pFRT2-Tel^r vectors have the same sequence. Depending on the orientation of *FRT* on the chromosome, either pFRT1-Tel^r or pFRT2-Tel^r would be used to orient the promoterless reporter fusion in the same direction as the promoter of interest. Abbreviations: *cI857*, temperature-sensitive repressor; *gfp*, green fluorescent protein gene; *lacZ*, β-galactosidase gene; *luxCDABE*, genes encoding the bacterial bioluminescent operon; T_1T_2, transcriptional terminators.

aided by the Flp-encoding helper plasmid, following mutant construction with an *FRT*-flanked antibiotic resistance cassette and Flp excision, has facilitated regulation studies of target genes at their native chromosomal loci (12, 37). In our experience, when coupled with *FRT*-based resistant-marker approaches for chromosomal mutagenesis (e.g., allelic replacement or *FRT*-based transposon) (32), these fusion vectors were found to be simple and powerful tools for studying gene regulation without promoter mapping or prior knowledge of promoter sequence or location. The disadvantages of previously reported fusion vectors and helper plasmids (12, 37) are the use of antibiotic resistance markers (Ap^r, Cm^r, Gm^r, Km^r, and Sp^r) and the limitation of a single reporter gene (*lacZ*). Here, the Tel^r cassette replaced the Gm^r cassette in the previously reported vectors pFRT1-*lacZ* and pFRT2-*lacZ* (37), resulting in pFRT1-*lacZ*-Tel^r and pFRT2-*lacZ*-Tel^r (Fig. 4B). The difference between these two new vectors is the orientation of the *FRT* sequence relative to that of the reporter gene, accounting for the selection of the appropriate fusion vector relative to the orientation of the "*FRT* scar" on the chromosome, thus aligning the reporter gene in the same direction as the promoter. To provide more reporter gene options, four other fusion vectors (pFRT1-*gfp*-Tel^r, pFRT2-*gfp*-Tel^r, pFRT1-*lux*-Tel^r, and pFRT2-*lux*-Tel^r) were constructed based on the *gfp* and *lux* operon reporters (Fig. 4C and D). We constructed an Flp-encoding helper plasmid (pCD13SK-Flp-*oriT-asd*$_{Ec}$) based on the *asd*$_{Ec}$ nonantibiotic selectable marker for plasmid maintenance in *E. coli* Δ*asd* strains (e.g., E463 and E1354) (Table 1 and Fig. 4A).

The regulation mechanisms of the *betBA* operon have been widely studied in a variety of organisms such as *E. coli* (13), *Pseudomonas putida* (14), and *Sinorhizobium meliloti* (27). Extensive characterization of the *betBA* operon in *E. coli* (13) has shown that this operon is regulated by osmolarity, tempera-

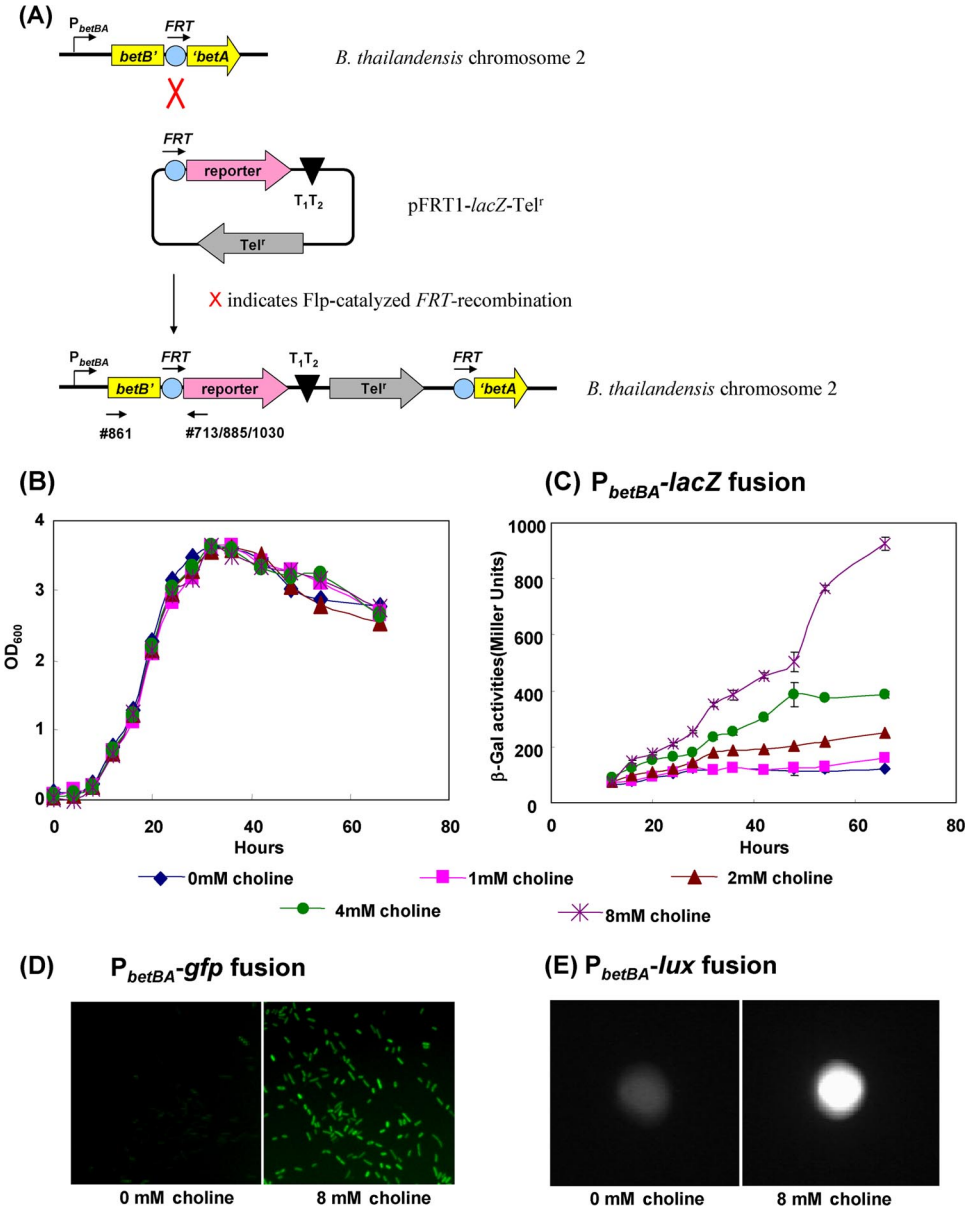

FIG. 5. Induction of the *betBA* operon by choline. (A) Flp-catalyzed recombination of the promoterless *FRT-lacZ* fusion into the *B. thailandensis* chromosome at the Δ*betBA*::*FRT* loci. Oligonucleotides 713 and 861 were used to screen for correct integration and are indicated by arrows. Oligonucleotides 885 and 1030 were used along with oligonucleotide 861 to screen *gfp* and *lux* reporters, respectively. (B) E264-Δ*betBA*::*FRT-lacZ* was grown in 1× M9 medium plus 20 mM glucose supplemented with 0, 1, 2, 4, or 8 mM choline. (C) β-Galactosidase assays were performed in triplicate for all of the growth cultures shown above (B) at various time points, indicating that *betBA* is inducible by choline. Two alternative fusion strains, E264-Δ*betBA*::*FRT-gfp*-Tel^r (D) and E264-Δ*betBA*::*FRT-lux*-Tel^r (E), were grown in MG medium or MG medium plus 8 mM choline. (D and E) The expressions of GFP (D) and bioluminescent proteins (E) were significantly induced in the presence of choline. Images in D are representative of multiple fields for the same samples. OD_{600}, optical density at 600 nm.

ture, oxygen, choline, and glycine betaine. However, little is known about the regulation of the *betBA* operon in *B. thailandensis*. As a proof of concept, we used one of the reporter fusion vectors to determine the regulatory mechanism of the *betBA* operon in *B. thailandensis* by choline and osmotic stress. *B. thailandensis* strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r was engineered using fusion vector pFRT1-*lacZ*-Tel^r and helper plasmid pCD13SK-Flp-*oriT-asd*$_{Ec}$ (Fig. 4A and B and 5A). Flp-catalyzed recombination and the orientation of the *FRT-lacZ* fusion at the "*FRT* scar" within the chromosome of the Δ*betBA* mutant, a location at which the *lacZ* reporter gene is controlled by an unknown *betBA* promoter, were verified by PCR using oligonucleotides 713 and 861 (Fig. 5A). Choline induction of the *betBA* operon was studied by growing fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r in 1× M9 medium with glucose and supplemented with different concentrations of choline. These conditions resulted in similar growth rates and overall cell densities (Fig. 5B). β-Galactosidase assays were performed

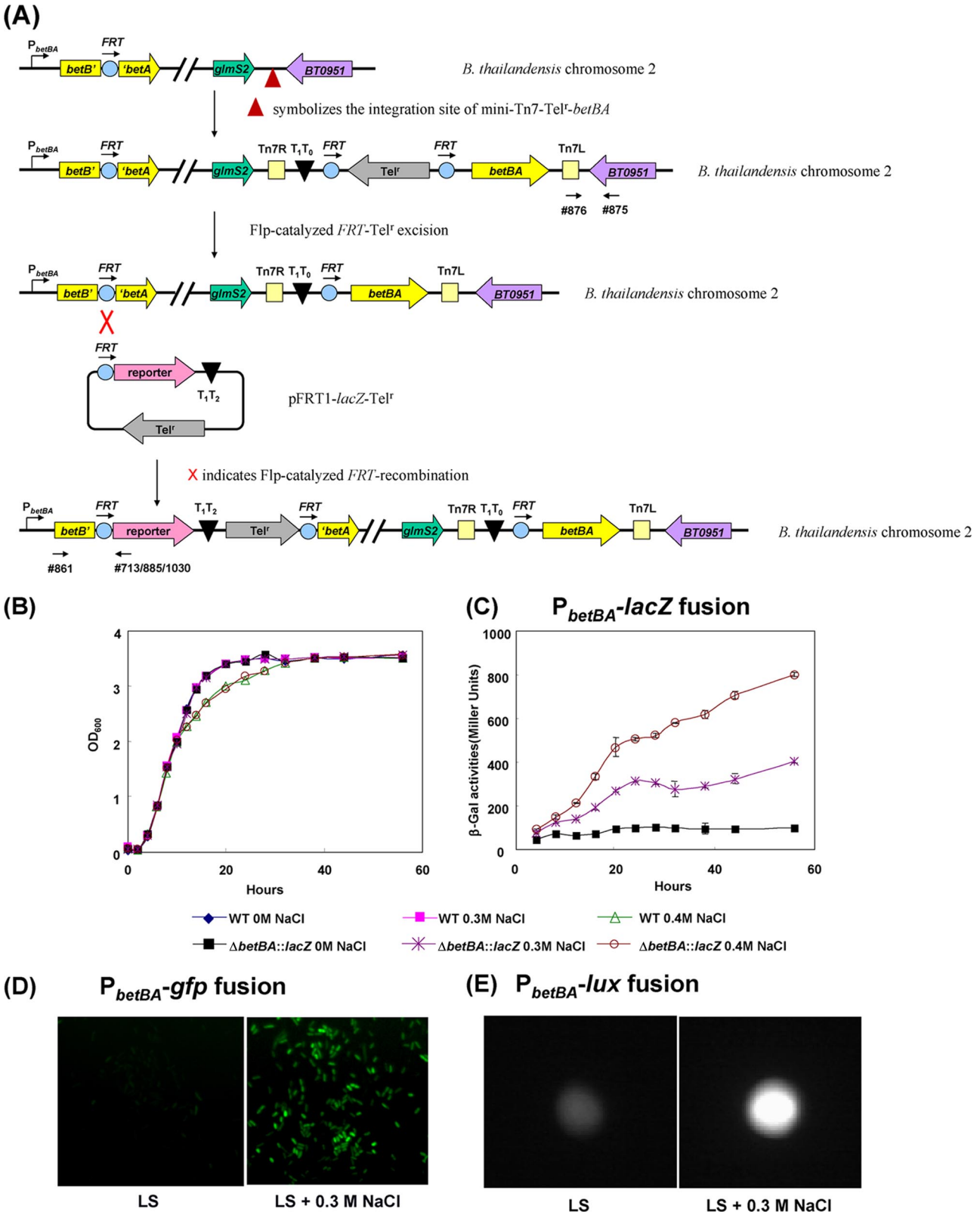

FIG. 6. Induction of the *betBA* operon by osmotic stress. (A) Strategy for constructing fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*. Oligonucleotide 861 was used along with oligonucleotide 713, 885, or 1030 for screening, and they are indicated by arrows. Parallel diagonal lines indicate a large distance of separation on the chromosome. (B) The resulting strain, E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*, and wild-type (WT) strain E264 were grown in LS (0 M NaCl) medium supplemented with 0.3 and 0.4 M NaCl. Both the wild-type and fusion strains produced identical growth characteristics in LS medium and LS medium plus 0.3 M NaCl yet displayed slightly decreased levels of growth in 0.4 M NaCl after mid-log phase. (C) At

to compare the expression levels of the *betBA* operon in different choline concentrations (Fig. 5C). As choline concentrations increased, corresponding increases in β-galactosidase activities were observed, indicating that the *betBA* operon was responsive to choline. Utilizing two alternative gene fusion vectors (pFRT1-*gfp*-Tel^r and pFRT1-*lux*-Tel^r), fusion strains E264-Δ*betBA*::*FRT-gfp*-Tel^r and E264-Δ*betBA*::*FRT-lux*-Tel^r were also constructed. By comparing the fluorescent and luminescent intensities, the induction of the *betBA* operon by choline was observed using the *gfp* or *lux* reporter, respectively (Fig. 5D and E).

A previously reported study has shown that the *E. coli betBA* operon was involved in osmotic regulation and was induced by osmotic stress (13). Here, we demonstrated that osmotic stress (e.g., NaCl) induces the *B. thailandensis betBA* operon by utilizing the mini-Tn*7*-Tel^r-based system and the reporter fusion vectors described above. To compare the level of expression of the *betBA* operon in the presence of osmotic stress to that in the absence of osmotic stress, it was necessary to obtain the same growth rates and overall cell densities through complementation of the Δ*betBA* mutation (Fig. 6B). Consequently, fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA* was constructed in the E264-Δ*betBA*::*FRT*/*attTn7*::*betBA* background (Fig. 6A) because we found that the growth of the Δ*betBA* mutant was significantly affected by NaCl without complementing the Δ*betBA* mutation. The constructed strain, E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA*, and wild-type strain E264 were grown in LS (0 M NaCl) medium supplemented with 0.3 and 0.4 M NaCl. These strains produced identical growth characteristics in LS medium and LS medium with 0.3 M NaCl, but both strains displayed slightly decreased growth rates in 0.4 M NaCl after the mid-log growth phase (Fig. 6B). The presence of 0.3 M NaCl significantly induced the *betBA* operon as determined by β-galactosidase assays (Fig. 6C). Despite the increased induction of the *betBA* operon in the presence of 0.4 M NaCl relative to that in the absence of NaCl, this comparison was unreliable due to differing growth rates. These data indicated that the *betBA* operon in *B. thailandensis* was induced by osmotic stress. Similarly, two fusion strains (E264-Δ*betBA*::*FRT-gfp*-Tel^r/*attTn7*::*betBA* and E264-Δ*betBA*::*FRT-lux*-Tel^r/*attTn7*::*betBA*) were constructed to show the induction of the *betBA* operon by observing the increased fluorescence and bioluminescence levels under conditions of osmotic stress (Fig. 6D and E).

DISCUSSION

In this study, the nonantibiotic Tel^r cassette was utilized to construct three genetic systems: a random mariner transposon, a mini-Tn*7* site-specific transposon vector, and six *FRT*-reporter fusion vectors based on three different reporters. A constitutive promoter, PC_{S12} (*B. cenocepacia rpsL* gene) (41), was included upstream of the Tel^r cassettes in all these tools to ensure the efficient expression of this resistance marker. First, the constructed *Himar1*-based random mariner transposon was successfully tested in four different *Burkholderia* spp.: *B. cenocepacia*, *B. thailandensis*, *B. pseudomallei*, and *B. mallei*. PCR screening with Tel-specific oligonucleotides revealed that 100% of Tel^r colonies harbored the Tel^r cassette. Next, the mini-Tn*7* system was utilized successfully to complement the Δ*betBA* mutant. Finally, three different *FRT*-reporter fusion vectors were used to study the regulation of the *B. thailandensis betBA* operon. Results showed that the *betBA* operon, which is essential for *B. thailandensis* choline degradation, was induced significantly by choline and osmotic stress (NaCl).

There are several advantages to including *FRT*-flanked resistance cassettes in random transposon mutagenesis and allelic replacement (2, 32). First, unmarked mutations can be obtained subsequent to allelic replacement or transposon mutagenesis by Flp excision of *FRT*-flanked resistance selection cassettes. The use of *FRT*-flanked resistance cassettes in allelic replacement allows for easier selection, resulting in higher mutation frequencies than that reported for a recently published approach to obtain unmarked mutations where there was a lack of positive selection for the second homologous recombination (15). Furthermore, the lack of positive selection requires laborious screening, and mutating essential genes may not be possible. In addition, the remaining "*FRT* scar" adds flexibility to subsequent fusion integrations, aiding in the construction of fusion strains for regulation studies of nonessential genes without prior knowledge of the identity and location of promoter sequences. For essential genes, mutant fusion strains can be complemented with the mini-Tn*7*-Tel^r system presented here, and gene regulation studies can be performed. Because single copies are more representative of the natural genetic regulation mechanism, as opposed to multicopy plasmids, single-copy tools could ameliorate the difficulties of complementation and promoter studies. The six *FRT*-reporter fusion vectors, based on three different reporters (*lacZ*, *gfp*, and the *luxCDABE* operon), add further flexibility and provide simplified visualization and quantification of gene expression during regulation studies. For example, by fusing the *gfp* reporter downstream of a target gene with pFRT-*gfp*-Tel^r vectors, it is possible to measure gene expression via detecting the bacterial green fluorescent protein (GFP) signal under different growth conditions or during eukaryotic cell infections. Similarly, by utilizing the pFRT-*lux*-Tel^r vectors, the regulation of target genes during animal model infections can be studied by measuring the bacterial bioluminescence intensity.

The genetic tools described in this paper will aid in elucidating the physiology, environmental behavior, and pathogenic mechanisms of *Burkholderia* species. Although the model or-

each time point, β-galactosidase assays were performed in triplicate on fusion strain E264-Δ*betBA*::*FRT-lacZ*-Tel^r/*attTn7*::*betBA* grown in LS medium with 0, 0.3, or 0.4 M NaCl. When the complemented fusion strain was grown under osmotically stressed conditions (0.3 and 0.4 M NaCl), the *betBA* operon was significantly induced, compared to conditions with no osmotic stress (no salt). (D and E) Two alternative fusion strains, E264-Δ*betBA*::*FRT-gfp*-Tel^r/*att*Tn*7*::*betBA* (D) and E264-Δ*betBA*::*FRT-lux*-Tel^r/*attTn7*::*betBA* (E), were grown in LS medium or LS medium plus 0.3 M NaCl. The expressions of GFP (D) and *luxCDABE* (E) were significantly induced in the presence of NaCl. Images in D are representative of multiple fields in the same sample. OD_{600}, optical density at 600 nm.

ganism *B. thailandensis* was utilized primarily to demonstrate the efficacy of these tools, Tel^r selection was successfully tested in the select agents *B. pseudomallei* and *B. mallei* by using the transposon pBT20-Δ*bla*-Tel^r-*FRT*. Thus, we believe that these tools could be particularly useful for various studies of *B. pseudomallei* and *B. mallei*. The alternative nonantibiotic asd_{Ec} selectable marker in helper plasmids pTNS3-asd_{Ec} and pCD13SK-Flp-*oriT*-asd_{Ec} may allow their use in other select-agent species. The genetic tools presented here could be further developed by substituting the Tel^r cassette with other nonantibiotic selectable markers. Finally, because these tools are completely devoid of any antibiotic resistance markers, they are in full compliance with CDC select-agent regulations.

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