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Genetic Tools for Allelic Replacement in *Burkholderia* Species▽†

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Allelic replacement in the *Burkholderia* genus has been problematic due to the lack of appropriate counter-selectable and selectable markers. The counter-selectable marker *sacB*, commonly used in gram-negative bacteria, is nonselective on sucrose in many *Burkholderia* species. In addition, the use of antibiotic resistance markers of clinical importance for the selection of desirable genetic traits is prohibited in the United States for two potential bioterrorism agents, *Burkholderia mallei* and *Burkholderia pseudomallei*. Here, we engineered a mutated counter-selectable marker based on the *B. pseudomallei* PheS (the α-subunit of phenylalanyl tRNA synthase) protein and tested its effectiveness in three different *Burkholderia* species. The mutant PheS protein effectively killed 100% of the bacteria in the presence of 0.1% *p*-chlorophenylalanine. We assembled the mutant *pheS* on several allelic replacement vectors, in addition to constructing selectable markers based on tellurite (Tel^r) and trimethoprim (Tp^r) resistance that are excisable by flanking unique FLP recombination target (FRT) sequences. As a proof of concept, we utilized one of these gene replacement vectors (pBAKA) and the Tel^r-FRT cassette to produce a chromosomal mutation in the *Burkholderia thailandensis betBA* operon, which codes for betaine aldehyde dehydrogenase and choline dehydrogenase. Chromosomal resistance markers could be excised by the introduction of pFLP-AB5 (Tp^r), which is one of two constructed *flp*-containing plasmids, pFLP-AB4 (Tel^r) and pFLP-AB5 (Tp^r). These *flp*-containing plasmids harbor the mutant *pheS* gene and allow self curing on media that contain *p*-chlorophenylalanine after Flp-FRT excision. The characterization of the Δ*betBA*::Tel^r-FRT and Δ*betBA*::FRT mutants indicated a defect in growth with choline as a sole carbon source, while these mutants grew as well as the wild type with succinate and glucose as alternative carbon sources.

Members of the *Burkholderia* genus are ubiquitous in the soil (i.e., rhizosphere) and water environments (31). Besides acting as saprophytes, several species associate beneficially with plants, while others cause plant diseases. Some (e.g., *Burkholderia cenocepacia*) are opportunists in human and animal infections. Others, such as *Burkholderia mallei* and *Burkholderia pseudomallei*, cause deadly infections in humans and animals (6, 33).

B. pseudomallei is the causative agent of melioidosis, an emerging global infectious disease, and this microbe is a potential bioterrorism agent of national biodefense concern. This disease has been predominantly studied, identified, and diagnosed in Thailand and northern Australia. However, melioidosis is considered endemic in many countries, including Thailand, Australia, Malaysia, Singapore, Vietnam, Burma, and possibly also Brunei, China, Hong Kong, Cambodia, Laos, India, and Taiwan (34). Cases have occurred in other areas of Asia, Africa, the Americas (e.g., Brazil and Puerto Rico), the Caribbean, the Middle East, and the Pacific (2, 9, 14, 15, 17, 21, 22, 24, 35). In the United States, *B. pseudomallei* and *B. mallei* (a clonal derivative of *B. pseudomallei*) are classified by the CDC as category B select agents because of the seriousness of the disease, the high fatality rates, the ease of isolation in soil throughout the tropics, and the historical use of the clonal derivative *B. mallei* during World Wars I and II by Germany (11).

The numerous genome sequences now available for several *Burkholderia* species, including the two potential bioterrorism agents, *B. mallei* and *B. pseudomallei*, should aid in the study to yield genetic, physiological, and pathogenic insights into members of this genus. For *B. mallei* and *B. pseudomallei*, the CDC restricts the use of resistance markers to clinically important antibiotics, which results in the need for non-antibiotic-selectable markers. Non-antibiotic-selectable markers based on Tel^r resistance could be useful for various *Burkholderia* spp., especially *B. mallei* and *B. pseudomallei*. The Tel^r marker, which is based on the genes *kilA*, *telA*, and *telB*, has been utilized successfully as a non-antibiotic-selectable marker in *Pseudomonas putida* and *Pseudomonas fluorescens* (16, 20, 26), and it could serve as an alternative selectable marker for *Burkholderia* species. For allelic replacement, Tel^r cassettes can be utilized only once without being coupled to the Flp recombination target (Flp-FRT) excision of the chromosomally located Tel^r-FRT cassette, which allows for the recycling of its use (13). Coupling the Tel^r selectable markers to the Flp-FRT system will allow numerous cycles of allelic replacement in *Burkholderia* spp. In addition, several counterselectable markers have been used for different bacteria, including *rpsL*, *tetAR*, *pheS*, *thyA*, *gata-1*, *ccdB*, and the most common, *sacB* (23). Although published works suggest that the *sacB* counter-selectable marker is suitable for some *Burkholderia* spp. (4, 5, 10, 30), it is an inappropriate and leaky counter-selectable marker for many laboratories, resulting in the need for an alternative counter-selectable marker. In addition, *rpsL*-based plasmids have been described

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for counter selection during allelic replacement (28), but such plasmids are limited to strains with chromosomal mutations in *rpsL* and the aminoglycoside efflux pump (7). The further development of non-antibiotic-selectable markers and counter-selectable markers should serve as useful genetic tools to perform molecular genetics, pathogenesis, and bacterium-host interaction studies for the discovery of novel vaccines, therapeutics, and diagnostic targets, as well as the environmental significance of various *Burkholderia* species.

This study describes novel genetic tools for making repetitive rounds of allelic replacements. A broad-host-range counter-selectable marker, together with several gene replacement vectors, has been engineered for use in *Burkholderia* species, and it will aid future allelic replacement experiments. Antibiotic and nonantibiotic resistance markers based on unique FRT sequences were developed for repetitive rounds of gene replacement, reducing the risks of undesirable deletions or genome rearrangements (1). These novel antibiotic and nonantibiotic FRT cassettes will be useful for gene replacement in both nonselect agents and category B select agents and should be useful in many laboratories that work on *Burkholderia* species of clinical and environmental importance.

MATERIALS AND METHODS

Bacterial strains, media, and culturing conditions. All strains and plasmids involved in this study are listed in Table 1. *Escherichia coli* EPMax10B (Bio-Rad) was routinely used as a cloning strain. *E. coli* strain EPMax10B-*pir116*-Δ*asd*::Gm (E1345) was used for the cloning of *asd*-complementing vectors (e.g., pBAKA; the *Pseudomonas aeruginosa asd* [asd_{Pa}] gene codes for aspartate semialdehyde dehydrogenase). The *E. coli* conjugal and suicidal strain EPMax10B-*pir116*-Δ*asd*-*mob*-Km-Δ*trp*::Gm (E1354) was used for plasmid mobilization into *B. thailandensis* through conjugation. Luria-Bertani (LB) medium (Difco) was used to culture all of the *E. coli* strains. *Burkholderia* strains (*B. thailandensis*, *B. cenocepacia* K56-2, and *B. dolosa*) and their derivatives were cultured in LB or 1× M9 minimal medium supplemented with 20 mM glucose. Alternative carbon sources (e.g., 20 mM succinate or 1% Casamino Acids) may be used; however, citrate should not be used, as we have observed a reduction in the number of CFU for some species when grown on citrate. *B. cenocepacia* J2315 and derivatives were cultured in LB or 1× M9 minimal medium containing 20 mM glucose and 0.125% (wt/vol) yeast extract. Antibiotics and nonantibiotic bacteriocides were added to the media for selection and plasmid maintenance as follows: for *E. coli*, Gm at 15 μg/ml, Ap at 110 μg/ml, Tel at 30 μg/ml, Tet at 10 μg/ml, Km at 35 μg/ml, and Tp at 50 μg/ml; for *B. thailandensis*, Km at 500 μg/ml, Tel at 125 μg/ml, and Tp at 300 μg/ml. Also, Tp at 200, 700, and 500 μg/ml were used for *B. cenocepacia* K56-2, *B. cenocepacia* J2315, and *B. dolosa*, respectively. For the growth of *E. coli* Δ*asd* strains EPMax10B-*pir116*-Δ*asd*::Gm and EPMax10B-*pir116*-Δ*asd*-*mob*-Km-Δ*trp*::Gm (E1345 and E1354 of Table 1), 100 μg/ml of diaminopimelic acid (DAP; Sigma) was supplied, unless the strain was complemented by the asd_{Pa} gene on the plasmid (e.g., pBAKA). DAP was dissolved in 1 M NaOH to make a 100-mg/ml stock solution. *E. coli* strain E1345 was used for all cloning steps, and strain E1354 was used for the mobilization of *oriT*-containing vectors by conjugation (Table 1). For strains E1345 and E1354, DAP (100 μg/ml) was added only during growth for competent cell preparation and the 1 h of recovery after transformation, but DAP was not added to medium plates for the selection of plasmid pBAKA and asd_{Pa}-containing plasmid derivatives. For the counter selection of *pheS*, 0.1% (wt/vol) *p*-chlorophenylalanine (cPhe; DL-4-chlorophenylalanine; Acros Organics) was autoclaved with the media.

Molecular methods and reagents. Unless otherwise indicated, 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 Zyppy Plasmid Miniprep kit I and Zymoclean gel DNA recovery kit, respectively, both from Zymo Research Corporation. Chemically competent cell (e.g., E1354) preparations and other molecular techniques were followed according to Sambrook and Russell (25). Oligonucleotide primers (Table 2) were synthesized by Integrated DNA Technologies. *Pfu* was purchased from Stratagene. Generally, we performed the various PCRs by initial denaturation for 1 min at 94°C and 34 cycles of 45 s at 94°C, 30 s at 58°C, and 1 min/kb at 72°C, and a final step of 10 min at 72°C was included.

Preparation of high-efficiency *E. coli* Δ*asd*-competent cells. For the cloning and maintaining of the asd_{Pa}-containing vectors (e.g., pBAKA), we prepared highly efficient *E. coli* Δ*asd* competent cells of strain E1345. *E. coli* Δ*asd* strain E1345 was grown in LB medium containing 15 μg/ml Gm (LB–Gm-15) and supplemented with DAP (100 μg/ml) overnight at 37°C. Next, the overnight culture was diluted 100-fold into 250 ml of fresh LB-DAP and shaken at 37°C and 250 rpm. When the optical density at 600 nm (OD_{600}) reached ~0.3 (at approximately 3 h), an equal volume (250 ml) of fresh prewarmed (37°C) LB-DAP–40 mM glucose was added to the growing culture. The culture was grown to an OD_{600} of ~0.7 (~1 h) and then transferred to ice. Cells were harvested immediately by centrifugation at 4°C and 8,000 rpm for 10 min. Supernatant was discarded, and cells were resuspended in a 1-ml total volume of 1 mM HEPES buffer (pH 7.0) and transferred to a chilled microcentrifuge tube. Cells were pelleted and washed four times with ice-cold 1 mM HEPES buffer (pH 7.0) in a refrigerated microcentrifuge at 9,000 rpm. The volume of cell pellets from the final spin were estimated, and the sample was gently resuspended in fresh 1 mM HEPES buffer with a 1/3 volume of the cell pellet size. A 40-μl volume of the competent cells was used for each electroporation. For long-term storage, the final concentration of 10% glycerol was added, and 45-μl aliquots of the competent cells were frozen at −80°C. When needed, frozen cells were thawed on ice prior to electroporation. All other *E. coli* strains could be prepared similarly to create highly competent cells.

Engineering of pUC57-*pheS* and construction of pBBR1MCS-Km-Tp and pBBR1MCS-Km-Tp-*pheS*. We submitted the mutated *pheS* gene sequence to GenScript Corporation, which synthesized and cloned the sequence into pUC57 to yield pUC57-*pheS*. This vector contains the mutated *B. pseudomallei pheS* gene with altered DNA sequences (see Fig. S3 in the supplemental material), which is driven by an upstream P_{S12} promoter of the *B. pseudomallei rpsL* gene. Using pUC57-*pheS*, we constructed pBBR1MCS-Km-Tp-*pheS* and pBBR1MCS-Km-Tp in several steps. First, pUC57-*pheS* was digested with SmaI and SacI, and the 1.1-kb P_{S12}-*pheS* fragment was cloned into pBBR1MCS-Km digested with the same enzymes, yielding pBBR1MCS-Km-*pheS*. Next, the 0.6-kb Tp^r cassette was obtained from pMLBAD by SalI and EcoRV digestion and was cloned into pBBR1MCS-Km-*pheS* digested with the same enzymes to construct pBBR1MCS-Km-Tp-*pheS*. Finally, pBBR1MCS-Km-Tp-*pheS* was digested with NdeI and XbaI and blunt ended, and the 5.8-kb backbone was self ligated to delete *pheS*, resulting in pBBR1MCS-Km-Tp.

Construction of gene replacement vectors. We constructed the gene replacement pBAKA vector in several steps, using the *P. aeruginosa* aspartate semialdehyde dehydrogenase (asd_{Pa}) gene as a non-antibiotic-selectable marker. asd_{Pa} was PCR amplified from pAM2B-P_{S12} using oligonucleotides 732 and 758, and the 1.1-kb asd_{Pa} fragment was digested with BamHI and BglII and ligated with pUC57-*pheS* that had been digested with BglII to construct pUC57-*pheS*-asd_{Pa}. This generated a construct with *pheS* and asd_{Pa} in the same orientation. Next, the pEX18Gm plasmid backbone was PCR amplified with oligonucleotides 826 and 463 to delete the *sacB*-Gm^r fragment, and the resulting 2.8-kb PCR product was digested with ScaI and BglII. The 2.2-kb *pheS*-asd_{Pa} fragment (removed from pUC57-*pheS*-asd_{Pa} with ScaI and BglII) was cloned into the pEX18Gm backbone, resulting in pEX18Gm-*pheS*-asd_{Pa}. Finally, pEX18Gm-*pheS*-asd_{Pa} was digested with BspHI and BglII, blunt ended, and self ligated to remove the remaining 0.4-kb Gm^r fragment, yielding pBAKA.

Five different gene replacement vectors, pEX18Ap-*pheS*, pEX18Gm-*pheS*, pEX18Km-*pheS*, pEX18Tc-*pheS*, and pEX18Tp-*pheS*, which harbored different antibiotic resistance cassettes, were constructed as described below. To replace the *sacB* gene with *pheS*, the plasmid backbones of pEX18Ap, pEX18Gm, and pEX18Tc were amplified with oligonucleotides 459 and 826. The *pheS* gene from pUC57-*pheS* (digested with ScaI and BglII and blunt ended) was ligated into these PCR products to create pEX18Ap-*pheS*, pEX18Gm-*pheS*, and pEX18Tc-*pheS*, respectively. For pEX18Tp-*pheS*, a larger 2-kb fragment, containing the dihydrofolate reductase gene that codes for Tp resistance, was amplified from pMLBAD with oligonucleotides 815 and 821. This PCR product was digested with NsiI and blunt ended. The 0.7-kb Tp^r cassette recovered from the gel was ligated into the pEX18Ap-*pheS* backbone, which was cut with BspHI and blunt ended to yield pEX18Tp-*pheS*. To create pEX18Km-*pheS*, pBBR1MCS-Km-Tp-*pheS* was digested with SspI and BglII and blunt ended, and the 1-kb Km^r fragment was cloned into the pEX18Gm-*pheS* backbone (digested with BsrGI and SacII and blunt ended), resulting in pEX18Km-*pheS*.

Construction of FRT vectors. pFRT plasmids, containing either a Tel^r or Tp^r marker flanked by two wild-type FRT genes, were constructed in this study. pPS856-ΔXbas was amplified with oligonucleotides 715 and 716, and then the 3.1-kb fragment was digested with EcoRV and XhoI and used as the plasmid

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Lab ID[a]	Relevant property(ies)	Source or reference
Strains			
E. coli			
EPMax10B	E1231	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*dlacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK rpsL nupG* λ$^-$	Bio-Rad
EPMax10B-*pir116*-Δ*asd*::Gm	E1345	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*dlacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK rpsL nupG* λ$^-$ *Tn-pir116-FRT2* Δ*asd*::Gm-FRT	–[b]
EPMax10B-*pir116*-Δ*asd-mob*-Km-Δ*trp*::Gm	E1354	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*dlacZ*Δ*M15* Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK rpsL nupG* λ$^-$ *Tn-pir116-FRT2* Δ*asd*::*FRT* Δ*trp*::Gm-FRT5 *mob*[*recA*::*RP4-2 Tc*::*Mu*-Km]	–[b]
B. thailandensis			
E264	E1298	Prototroph; environmental isolate	3
E264-Δ*betBA*::Tel^r-FRT	E1669	Tel^r; E264 with Tel^r cassette inserted in *betBA* operon	This study
E264-Δ*betBA*::FRT	E1671	*B. thailandensis* Δ*betBA*::FRT mutant	This study
B. cenocepacia K56-2	E1554	Prototroph; cystic fibrosis isolate	P. Sokol
B. cenocepacia J2315	E1553	Prototroph	J. Goldberg
B. dolosa AU0158	E1551	Prototroph	J. Goldberg
Plasmids			
pUC57-*pheS*	E1510	Ap^r; cloning vector harboring engineered *pheS*	This study
pBBR1MCS-Km	E1277	Km^r; broad-host-range cloning vector	18
pBBR1MCS-Km-*pheS*	E1511	Km^r; engineered *pheS* cloned into pBBR1MCS-Km	This study
pBBR1MCS-Km-Tp-*pheS*	E1558	Km^r, Tp^r; Tp^r cassette cloned into pBBR1MCS-Km-*pheS*	This study
pBBR1MCS-Km-Tp	E1579	Km^r, Tp^r; *pheS* removed from pBBR1MCS-Km-Tp-*pheS*	This study
pMLBAD	E1642	Tp^r; cloning vector containing Tp^r cassette	19
pAM2B-P_{S12}	E1297	asd_{Pa} and P_{S12}-*merTPA*-containing vector	–[b]
pUC57-*pheS*-asd_{Pa}	E1608	Ap^r; asd_{Pa} cloned into pUC57-*pheS*	This study
pEX18Gm-*pheS*-asd_{Pa}	E1614	Ap^r; *pheS*-asd_{Pa} cloned into pEX18Gm backbone	This study
pBAKA	E1624	Gene replacement vector based on asd_{Pa}	This study
pBAKA-*betBA*	E1628	*B. thailandensis betBA* operon cloned into pBAKA	This study
pBAKA-Δ*betBA*::Tel^r-FRT	E1640	Tel^r-FRT cassette inserted into *betBA* operon	This study
pEX18Ap	E0055	Ap^r; *sacB*-based gene replacement vector	13
pEX18Ap-*pheS*	E1618	Ap^r; gene replacement vector based on *pheS* and Ap^r	This study
pEX18Gm	E0062	Gm^r; *sacB*-based gene replacement vector	13
pEX18Gm-*pheS*	E1616	Gm^r; gene replacement vector based on *pheS* and Gm^r	This study
pEX18Km-*pheS*	E1753	Km^r; gene replacement vector based on *pheS* and Km^r	This study
pEX18Tet	E0064	Tet^r; *sacB*-based gene replacement vector	13
pEX18Tet-*pheS*	E1620	Tet^r; gene replacement vector based on *pheS* and Tet^r	This study
pEX18Tp-*pheS*	E1653	Tp^r; gene replacement vector based on *pheS* and Tp^r	This study
pPS856-ΔXbas	E1044	Gm^r, Ap^r; Gm^r cassette flanked by wild-type FRTs	–[b]
pBTB-6	E1508	Tel^r; Tel^r cassette containing plasmid	20
pBTB-SDM	E1555	Tel^r; pBTB-6 with SmaI, XhoI, and EcoRI sites mutated	This study
pwFRT-Mer^r	E1557	Mer^r, Ap^r; Mer^r cassette flanked by wild-type FRT	–[b]
pwFRT-Tel^{rc}	E1584	Tel^r, Ap^r; Tel^r cassette flanked by wild-type FRT	This study
pwFRT-Tp^{rc}	E1659	Tp^r, Ap^r; Tp^r cassette flanked by wild-type FRT	This study
pFLP2	E0067	Ap^r; *flp*-containing plasmid	13
pFLP-AB2a	E1565	Tel^r, *sacB*$^+$; pFLP2 with Ap^r replaced with Tel^r cassette	This study
pFLP-AB4a	E1569	Tel^r, *pheS*$^+$; *sacB* on pFLP-AB2a replaced with *pheS*	This study
pFLP-AB4	E1661	Tel^r; Flp-containing plasmid with mutated Tel^r cassette	This study
pFLP-AB5	E1662	Tp^r; pFLP-AB4 with Tel^r replaced with Tp^r cassette	This study

[a] Please use the laboratory identification number (Lab ID) when requesting strains and plasmids.
[b] Details on the engineering of these strains and plasmids are to be published elsewhere.
[c] Four other FRT mutants exist for each of these plasmids, in which each plasmid has the identical FRT spacer sequence flanking each selectable marker and the only sequence differences in the mutant plasmids (pmFRT-Tel^r, pmFRT-Tp^r, pFRT1-Tel^r, pFRT1-Tp^r, pFRT2-Tel^r, pFRT2-Tp^r, pFRT3-Tel^r, and pFRT3-Tp^r) relative to the sequences of pwFRT-Tel^r and pwFRT-TelTp^r are within the spacer sequence of each FRT.

backbone for the construction of pwFRT-Tel^r (wFRT indicates the wild-type FRT). Site-directed mutagenesis was performed on the Tel^r cassette (*kilA telAB*) of pBTB-6 with oligonucleotides 827, 828, and 829, resulting in pBTB-SDM, which had the SmaI, XhoI, and EcoRI sites removed from the *telA* and *telB* genes. Next, two-step PCR was performed to introduce the sequence of the promoter of the *B. cenocepacia rpsL* gene (PC_{S12}) upstream of the Tel^r cassette. Oligonucleotides 836 and 834 were used in the first PCR to amplify the Tel^r cassette from pBTB-SDM. This yielded a 3.1-kb PCR product, which was used as a template for the second PCR with oligonucleotides 837 and 834. The final product, with PC_{S12} upstream of the Tel^r cassette, was digested with XhoI and ligated into the pPS856-ΔXbas backbone as described above to yield pwFRT-Tel^r.

The construction of pwFRT-Tp^r was based on a laboratory plasmid, pwFRT-Mer^r. PCR was performed on pMLBAD using oligonucleotides 815 and 821 to amplify a larger Tp^r cassette, and the 2-kb fragment was digested with EcoRV. The smaller 0.6-kb Tp^r cassette was 5′ phosphorylated with T4 polynucleotide kinase and ligated into the pwFRT-PC_{S12} backbone (which was amplified from pwFRT-Mer^r by using oligonucleotides 792 and 715). The orientation of the Tel^r and Tp^r cassettes relative to the PC_{S12} promoter on plasmids pwFRT-Tel^r and pwFRT-Tp^r was confirmed by restriction mapping and DNA sequencing.

Besides these two pwFRT plasmids (pwFRT-Tel^r and pwFRT-Tp^r) with TCTAGAAA as the core spacer of the FRT sequences, we also constructed eight other plasmids of these two resistance markers based on four other unique FRT sequences: pmFRT-Tel^r and pmFRT-Tp^r, with the flanking FRT spacer TGTA

TABLE 2. Oligonucleotide primers used in this study

Primer no. and name	Sequence[a]
459; *sacB*-end	5′-CAACGTTTGCGCCTAGCTTC-3′
463; GmR-RT	5′-GAGCAGCCGCGTAGTGAG-3′
715; pPS854-XhoI	5′-AAGCTCGAGCTAATTCC-3′
716; pPS854-Cla-EcoRV	5′-CAATATCGATATCCATTGCTGTTGACAAAG-3′
732; asd_{Pa}-BglII	5′-CAATAGATCTCCGATCAGCGCTCCAGCAG-3′
758; asd_{Pa}-BamHI	5′-ATGAGGATCCAGTTGCGATGAAGCGTGTAGG-3′
792; *merT*-reverse	5′-CAAGCCCTCCAGTGAAGA-3′
815; pBAD-ScaI	5′-CTGTAGTACTCCAAAAAAACGGGTATGGAGA-3′
821; *dhfr*-SpeI	5′-ACGCACTAGTGGAACGAAATCGATGAG-3′
826; *oriT*-ScaI	5′-GCTTGCCCTCAGTACTGTTACGCCGGCG-3′
827; *tel*-ΔSmaI	5′-GGGAACGACCCTGGCCGCGTGCA-3′
828; *tel*-ΔXhoI	5′-GTAGGCGGCCTGGAGGCCCGAG-3′
829; *tel*-ΔEcoRI	5′-TCGGGGGTGAGTTCTGCGGGTT-3′
834; *tel*-XhoI	5′-CCTCCTCGAGCAGAAAGTCAAAAGCCTC-3′
836; *tel*-start	5′-CTTTAAGAAGGAGATATACCATGGAAGAACAAAGCGTGAA-3′
837; PC_{S12}	5′-ATCAGCCGTTGACTTAGTTGGTATTTCCGGAATATCATGCTGG GTTCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATACC-3′
861; Bt-*betB*-HindIII	5′-CCCGCAAGCTTGCCGGCAA-3′
862; Bt-*betA*-KpnI	5′-GACCGGTACCCGGCGGGCGGGGATAT-3′
867; Bt-*betB*-up	5′-GCACATCAAGCCGGACCAG-3′
868; Bt-*betA*-down	5′-CCGGGCCGAATATCGACGG-3′

[a] Restriction enzyme sites utilized in this study are underlined.

GATA; pFRT1-Tel^r and pFRT1-Tp^r, with the TCTTGAAA spacer; pFRT2-Tel^r and pFRT2-Tp^r, with the TCTAGGAA spacer; and pFRT3-Tel^r and pFRT3-Tp^r, with the TCTCGAAA spacer. The underlined bases represent differences in the core spacer sequence yielding unique FRT sequences. These 10 resistance plasmids (pwFRT-Tel^r, pwFRT-Tp^r, pmFRT-Tel^r, pmFRT-Tp^r, pFRT1-Tel^r, pFRT1-Tp^r, pFRT2-Tel^r, pFRT2-Tp^r, pFRT3-Tel^r, and pFRT3-Tp^r) are useful for performing multiple allelic replacement and Flp-FRT excision by using and recycling the same resistance marker without the risk of undesirable genomic deletions or inversions. To construct these eight plasmids, laboratory vectors pmFRT-Gm^r, pFRT1-Gm^r, pFRT2-Gm^r, and pFRT3-Gm^r were PCR amplified with oligonucleotides 715 and 716 to obtain plasmid backbones without the Gm^r marker. Each plasmid backbone was digested with EcoRV and XhoI, and the Tel^r and Tp^r fragments that were digested with the upstream PC_{S12} promoter were removed from pwFRT-Tel^r and pwFRT-Tp^r, respectively, with EcoRV and XhoI. These resistance fragments were individually cloned into the EcoRV and XhoI sites of each FRT plasmid backbone. Essentially, the sequences of all four new Tel^r-FRT plasmids are the same as that for pwFRT-Tel^r, with the exception of the FRT spacer sequence flanking the resistant cassette; this is also the case for all Tp^r-FRT plasmids.

Construction of *flp*-containing plasmids. For the excision of chromosome-selectable markers in mutant strains, we engineered two *flp*-containing vectors, pFLP-AB4 (Tel^r) and pFLP-AB5 (Tp^r). First, we constructed pFLP-AB4a by basing it on the Tel-resistant cassette. The Tel^r cassette, including *kilA*, *telA*, and *telB* genes, was amplified from pBTB-6 by using oligonucleotides 834 and 836. The blunt-ended 3.1-kb fragment was 5′ phosphorylated and ligated into pFLP2, which was digested with SspI and BsaI and blunt ended, to yield pFLP-AB2a. Next, pFLP-AB2a was digested with XbaI and SphI, and the *pheS* gene from pUC57-*pheS* was removed with the same enzymes and cloned into this plasmid, resulting in pFLP-AB4a. The PC_{S12}-Tel^r (3.1 kb) or PC_{S12}-Tp^r (0.6 kb) fragment was obtained from pwFRT-Tel^r or pwFRT-Tp^r by EcoRV and XhoI digestion and then was cloned into the pFLP-AB4a backbone (cut with NcoI, blunt ended, and digested with XhoI). These resulted in pFLP-AB4 and pFLP-AB5, which contain the PC_{S12} promoter driving the Tel^r or Tp^r cassette, respectively.

Construction of pBAKA-Δ*betBA*::Tel^r-FRT. The *B. thailandensis betBA* operon was amplified from chromosomal DNA of strain E264 by using oligonucleotides 861 and 862 (see Fig. 4A). The 4.5-kb fragment was digested with HindIII and KpnI and cloned in pBAKA that had been digested with the same enzymes, yielding pBAKA-*betBA*. Next, pBAKA-*betBA* was digested with SmaI, and the 6.6-kb backbone was ligated with the SmaI fragment (Tel^r-FRT) from pwFRT-Tel^r, resulting in pBAKA-Δ*betBA*::Tel^r-FRT. For these gene replacement vectors, a 2.2-kb internal region of *betBA* on pBAKA-*betBA* was deleted, and the Tel^r-FRT cassette is in the same orientation as the *betBA* operon (see Fig. 4A).

Engineering of unmarked *B. thailandensis* Δ*betBA*::FRT mutants. *E. coli* strain EPMax10B-*pir116*-Δ*asd*-*mob*-Km-Δ*trp*::Gm (E1354) was used as the conjugal donor to introduce the gene replacement vector pBAKA-Δ*betBA*::Tel^r-FRT into *B. thailandensis* strain E264. Both recipient and donor strains were grown to log phase prior to conjugation. Conjugation was performed by mixing 0.5 ml of each of the donor and recipient strains at approximately equal cell densities. The tube was centrifuged at 9,000 × *g* for 1 min, and all of the supernatant was discarded, except for 30 μl, which was used to gently resuspend the cell pellet. The 30-μl mixture of cell suspension was spotted onto cellulose acetate filters (Sartorius), which were prewarmed at 37°C on LB agar plates, and the conjugation plates were incubated at 37°C for 8 h. Conjugation filters were aseptically transferred to a 1.5-ml microcentrifuge tube and vortexed in 1 ml of 1× M9 minimal medium to resuspend cells. One hundred microliters and equal volumes of 10× dilutions were plated on plates containing 1× M9 medium supplemented with 20 mM glucose and 125 μg/ml Tel (Tel-125). Resulting colonies were streaked out on 1× M9 minimal medium–20 mM glucose–0.1% cPhe–Tel-75. Tel^r mutants were screened by being patched with toothpicks onto plates of MPG (1× M9 minimal medium–0.1% cPhe–20 mM glucose) and MPC (M9 minimal medium–0.1% cPhe–20 mM choline chloride [Sigma]). Mutants growing on glucose but defective in choline degradation were purified once on MPG medium and further screened through PCR using oligonucleotides 867 and 868 (see Fig. 4A), which anneal upstream and downstream of the *betBA* operon and outside of oligonucleotides 861 and 862, which were used for cloning.

As a proof of concept, we performed Flp excision on the chromosomal Tel^r marker from the *B. thailandensis* Δ*betBA*::Tel^r-FRT mutant. To perform Flp excision on the Tel^r cassette in the Δ*betBA*::Tel^r-FRT mutant, pFLP-AB5 was introduced into *B. thailandensis* mutant strain E264/Δ*betBA*::Tel^r-FRT through conjugation. The conjugation of E1354, harboring pFLP-AB5, with E264/Δ*betBA*::Tel^r-FRT was performed as described above for allelic replacement. Note that DAP is required when this *E. coli* strain was used to mobilize pFLP-AB4 and pFLP-AB5, but no DAP is required after conjugation, leading to DAP-less death. After conjugation, cells were plated on 1× M9 minimal medium–20 mM glucose–300 μg/ml Tp (1× M9 minimal medium–20 mM glucose–Tp-300) to select for single colonies of E264/Δ*betBA*::Tel^r-FRT containing pFLP-AB5. pFLP-AB5 then was cured by streaking these single Tel^r colonies on 1× M9 minimal medium–20 mM glucose–0.1% cPhe. Next, colonies from the cPhe plates were patched onto 1× M9 minimal medium–20 mM glucose with or without Tel-75 to confirm the excision of the Tel^r cassette, in addition to being patched on a 1× M9 minimal medium–20 mM glucose–Tp-300 plate to ensure that pFLP-AB5 was cured. Similarly, pFLP-AB4 could be used to excise FRT cassettes other than Tel^r-FRT. Finally, the phenotype of the *B. thailandensis* Δ*betBA*::FRT strain was confirmed by patching it onto 1× M9 minimal medium supplemented with 20 mM glucose or choline chloride along with wild-type strain E264 as a positive control. The inactivation of the *betBA* operon was characterized by the inability to grow on choline as a sole carbon source.

Characterization of Δ*betBA* mutants. The *B. thailandensis* Δ*betBA*::Tel^r-FRT and Δ*betBA*::FRT mutants were chosen to be further characterized by growth curve analyses, and the results were compared to those for wild-type *B. thailan-*

densis with choline, succinate, or glucose as the sole carbon source. These strains were grown overnight at 37°C in LB medium. Overnight cultures were washed once with one volume of 1× M9 minimal medium and resuspended in equal volumes of the same buffer. Resuspended cultures then were diluted 100-fold into fresh 1× M9 minimal medium containing 30 mM succinate, 20 mM glucose, or 30 mM choline chloride and grown at 37°C. At each time point, a measurement was taken at an OD_{600} for each culture.

Nucleotide sequence accession numbers. All of the vectors presented in Fig. 2 and 3 were submitted to GenBank. The accession numbers are the following: pUC57-*pheS*, EU277853; pBAKA, EU277854; pEX18Ap-*pheS*, EU332346; pEX18Gm-*pheS*, EU332347; pEX18Km-*pheS*, EU491017; pEX18Tc-*pheS*, EU332348; pEX18Tp-*pheS*, EU334848; pwFRT-Tel^r, EU329006; pwFRT-Tp^r, EU334849; pFLP-AB4, EU329004; and pFLP-AB5, EU334847.

RESULTS AND DISCUSSION

Characterization of the *B. pseudomallei* mutant *pheS* as a counter-selectable marker. The lack of an appropriate counter-selectable marker for a broader range of *Burkholderia* species and strains has hampered various molecular genetic studies of this genus. Existing tools for allelic replacement based on *rpsL* and aminoglycoside efflux pump mutations (7, 28), although useful, have been limited to specific strains. Previously, the utilization of the mutant (A294G) *E. coli pheS* gene was successful for allelic replacement in *E. coli* (12). Recent data from our laboratory (unpublished) indicate that the *P. aeruginosa pheS* gene carrying a similar mutation (A305G) killed *P. aeruginosa* in the presence of cPhe. However, we observed that neither the *E. coli* nor the *P. aeruginosa* mutant *pheS* gene efficiently killed *Burkholderia* species in the presence of cPhe (data not shown). We reasoned that the interactions between PheS (α-subunit) and PheT (β-subunit) of the functional multisubunit complex phenylalanyl tRNA synthase could be genus or species specific. The alignment of various PheS proteins from different *Burkholderia* species indicated high amino acid identity (93 to 97%) between members of this genus (see Fig. S1 in the supplemental material), suggesting that the interactions between PheS and PheT in different *Burkholderia* species are highly conserved. Thus, the similar A304G mutation of a *Burkholderia* (e.g., *B. pseudomallei*) *pheS* gene may be widely applicable for this genus. However, the high level of conservation of the *pheS* gene among the *Burkholderia* species at the DNA level (see Fig. S2 in the supplemental material) may present problems such as an undesirable homologous recombination at the *pheS* locus during allelic replacement. Therefore, we engineered a new *B. pseudomallei pheS* gene by switching alternative codons that existed on the same protein while conserving the amino acid sequence and introducing the critical A304G mutation (see Fig. S3 in the supplemental material). Although not yet tested, we reasoned that this strategy alters the DNA sequence of the *B. pseudomallei pheS* gene significantly enough to possibly reduce aberrant and undesirable recombination during allelic replacement at this locus.

To initially test the effectiveness of the engineered *B. pseudomallei* mutant *pheS* in other *Burkholderia* species, we constructed two replicative plasmids, pBBR1MCS-Km-Tp and pBBR1MCS-Km-Tp-*pheS*. Plasmid pBBR1MCS-Km-Tp-*pheS* was transformed into *B. cenocepacia* K56-2, *B. dolosa*, *B. cenocepacia* J2315, and *B. thailandensis* to test for killing on cPhe. The results indicate that the engineered *B. pseudomallei pheS* mutant effectively killed 100% of cells (~50,000 to 100,000 CFU) that were plated on medium containing 0.1% cPhe (Fig. 1), demonstrating its effectiveness as a counter-selectable marker. In addition, no spontaneous cPhe-resistant colonies were observed for strains harboring the mutant *pheS* gene, even after 2 weeks of incubation.

Construction of gene replacement vectors and FRT-based selectable markers. We next constructed several gene replacement vectors based on the *B. pseudomallei* mutant *pheS* gene (Fig. 2). Plasmids pEX18Ap-*pheS*, pEX18Gm-*pheS*, pEX18Km-*pheS*, pEX18Tc-*pheS*, and pEX18Tp-*pheS* will be useful for allelic replacement in various *Burkholderia* species. However, for the two restricted category B select agents, *B. mallei* and *B. pseudomallei*, we constructed pBAKA to prevent the further introduction of antibiotic resistance into these two organisms. Instead of antibiotic resistance selection, pBAKA contains the asd_{Pa} gene (encoding aspartate semialdehyde dehydrogenase from *P. aeruginosa*) as a selectable marker for the cloning and manipulation of *E. coli* Δ*asd* strains. The Δ*asd E. coli* cloning and mobilizable strains E1345 and E1354 (Table 1), respectively, were routinely used in our laboratory for pBAKA and other *asd*-containing plasmids. All allelic replacement vectors contain the *B. pseudomallei* mutant *pheS* gene driven by P_{S12}, the promoter of the *B. pseudomallei rpsL* gene, which was previously described (36). The addition of this conserved *rpsL* promoter sequence (AGCTGTTGACTCGC TTGGGATTTTCGGAATATCATGCCGGGT; the −35 and −10 regions are underlined) ensures the sufficient expression of the mutant *pheS* in *Burkholderia* species to outcompete the native chromosomal copy for PheT.

We engineered two selectable markers to contain flanking FRT sequences, which will be useful for *Burkholderia* species (Fig. 3A to C). FRT cassettes have been very beneficial for multiple rounds of allelic replacement and the recycling of useful selectable markers (8, 13). Therefore, we constructed pwFRT-Tel^r and pwFRT-Tp^r, which encode resistance to Tel and Tp, respectively (Fig. 3A and B). Unique restriction sites flanking these cassettes allow easy manipulations, and site-directed mutagenesis was performed to eliminate all repetitive restriction sites from within these resistance cassettes (see Materials and Methods). Tp resistance has been shown to be useful for various *Burkholderia* species (27). However, for restricted category B select agents (e.g., *B. mallei* and *B. pseudomallei*), the Tel^r-FRT cassette will be more appropriate for preventing the engineering of strains resistant to clinically important antibiotics. Each of the two cassettes is driven by the *B. cenocepacia rpsL* PC_{S12} promoter (AGCCGTTGACTTAGTTG GTATTTCCGGAATATCATGCTGGGT), with the −35 and −10 underlined regions conserved from the *B. pseudomallei* P_{S12} promoter described above. However, the intervening sequences between these two regions of the PC_{S12} and P_{S12} promoters are very different (36), which prevents any possible recombination between these two promoter sequences during allelic replacement.

Although the Flp excision of FRT cassettes is useful for recycling selectable markers for subsequent rounds of allelic replacement (13), each round of mutation and Flp excision results in one chromosomal FRT scar at the replaced locus (Fig. 4A). As a result, a previous study showed that multiple rounds of sequential allelic replacement with an identical wild-type FRT could lead to the Flp-catalyzed inversion of large chromosomal pieces (1). In addition, creating multiple closely linked mutations with one identical FRT cassette yielded Flp-

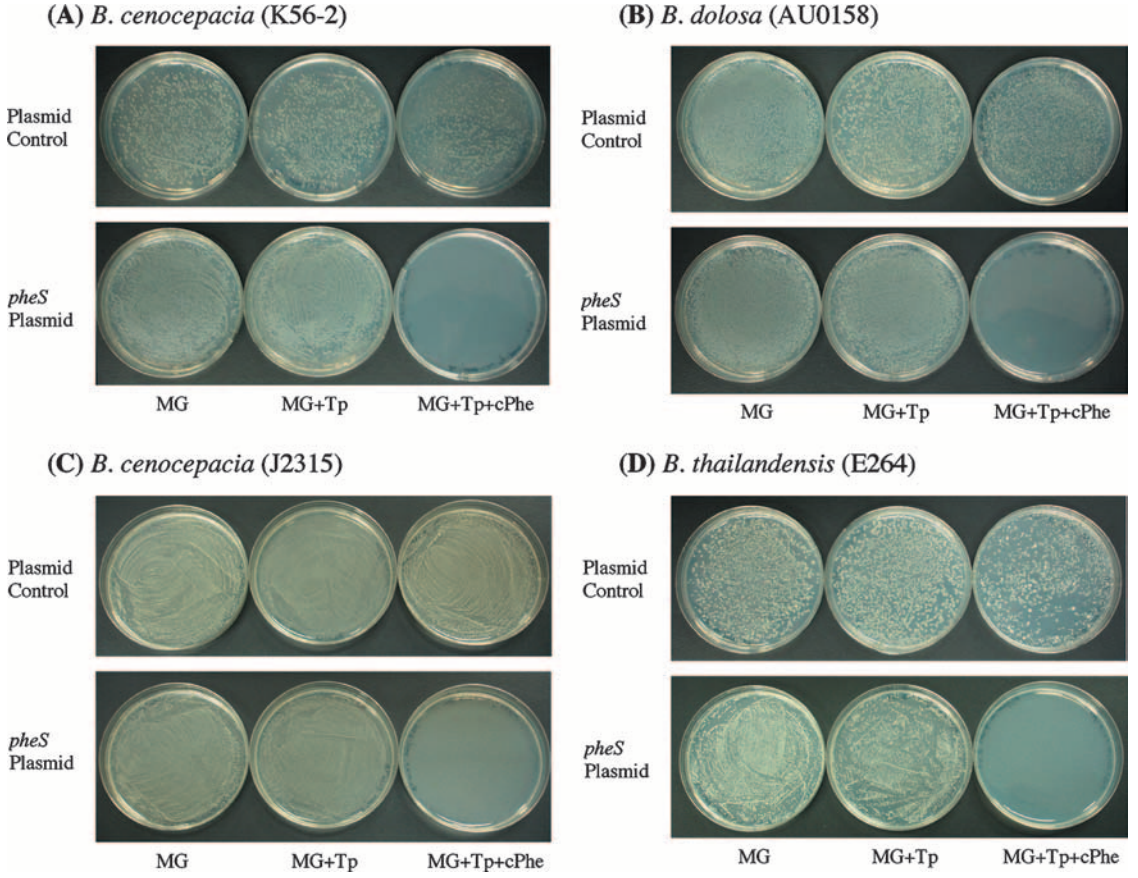

FIG. 1. Killing of four different *Burkholderia* species by a *pheS* plasmid (pBBR1MCS-Km-Tp-*pheS*) in the presence of 0.1% cPhe. The control plasmid used was pBBR1MCS-Km-Tp. Tp was used at 200 μg/ml for *B. cenocepacia* K56-2 (A), 500 μg/ml for *B. dolosa* (B), and 700 μg/ml for *B. cenocepacia* J2315 (C); kanamycin (Km) was used at 500 μg/ml for *B. thailandensis* (D). The same numbers of CFU were plated for each strain on three different media, 1× M9–glucose medium (MG), 1× M9–glucose medium containing Tp (MG+Tp), and 1× M9–glucose medium containing Tp and cPhe (MG+Tp+cPhe); more bacteria were plated in the bottom picture for each species to show effective killing at a high number of CFU. The four *Burkholderia* species containing the mutant *pheS* died in the presence of cPhe. No synergistic effect of Tp/Km and cPhe was observed. There was no effect on the number of CFU when each control strain was grown on cPhe-Tp or cPhe-Km.

catalyzed deletions of chromosomal genes between the two loci (unpublished data). Since wild-type Flp protein does not recombine FRT sequences of different spacer sequences and only recombines identical FRT sequences (29, 32), we have utilized unique FRT sequences with altered core spacer sequences, which prevented undesirable deletions of chromosomal fragments in multiple loci allelic replacements (unpublished data). We have used other resistance markers based on these unique FRT sequences for multiple mutations in several species, including *E. coli*, *Sphingomonas chlorophenolica*, and *P. aeruginosa*, without any undesirable deletions or rearrangements (unpublished data). Accordingly, to prevent undesirable chromosomal deletions and rearrangements, we have engineered each of the Tel^r and Tp^r cassettes flanked by five unique sets of FRTs (Fig. 3A and B; also see Materials and Methods). Therefore, when performing multiple allelic replacements on the chromosomes of the same bacteria, we recommend using Tel^r or Tp^r cassettes flanked by unique FRTs, which allows for multiple rounds of allelic replacement and the recycling of these resistance determinants.

Engineering of *B. thailandensis* Δ*betBA*::Tel^r-FRT chromosomal mutants. As a proof of concept of the developed system, we constructed a deletion in the *B. thailandensis betBA* operon by using pBAKA and the Tel^r-FRT cassette. pBAKA-Δ*betBA*::Tel^r-FRT was constructed in *E. coli* strain E1345. These plasmids were transformed into another *E. coli* strain, E1354, and mobilized into *B. thailandensis* by routine conjugation, as described in Materials and Methods. The selection of exconjugants (merodiploids) (Fig. 4A) was performed on 1× M9 minimal medium–20 mM glucose–Tel-125. The *E. coli* conjugal donor strain E1354 cannot grow on this minimal medium due to the lack of tryptophan, and we observed no *E. coli* in the background. We routinely obtained 100% of mutants after the counter-selection step on cPhe. However, it is critical that the counter-selection medium, in the presence of cPhe, contains no competing phenylalanine for clean counter selection. Other saccharides or succinate could be substituted for glucose if required. For fastidious *Burkholderia* species requiring amino acids in addition to 1× M9 minimal medium–20 mM glucose, the addition of 1 mM amino acid mix lacking phenylalanine and tryptophan often is appropriate for *pheS* counterselection. Alternatively, 1.25 g/liter of yeast extract, in addition to 1× M9 minimal medium–20 mM glucose, works well for fastidious *Burkholderia* species (e.g., *B. cenocepacia*). The initial screen-

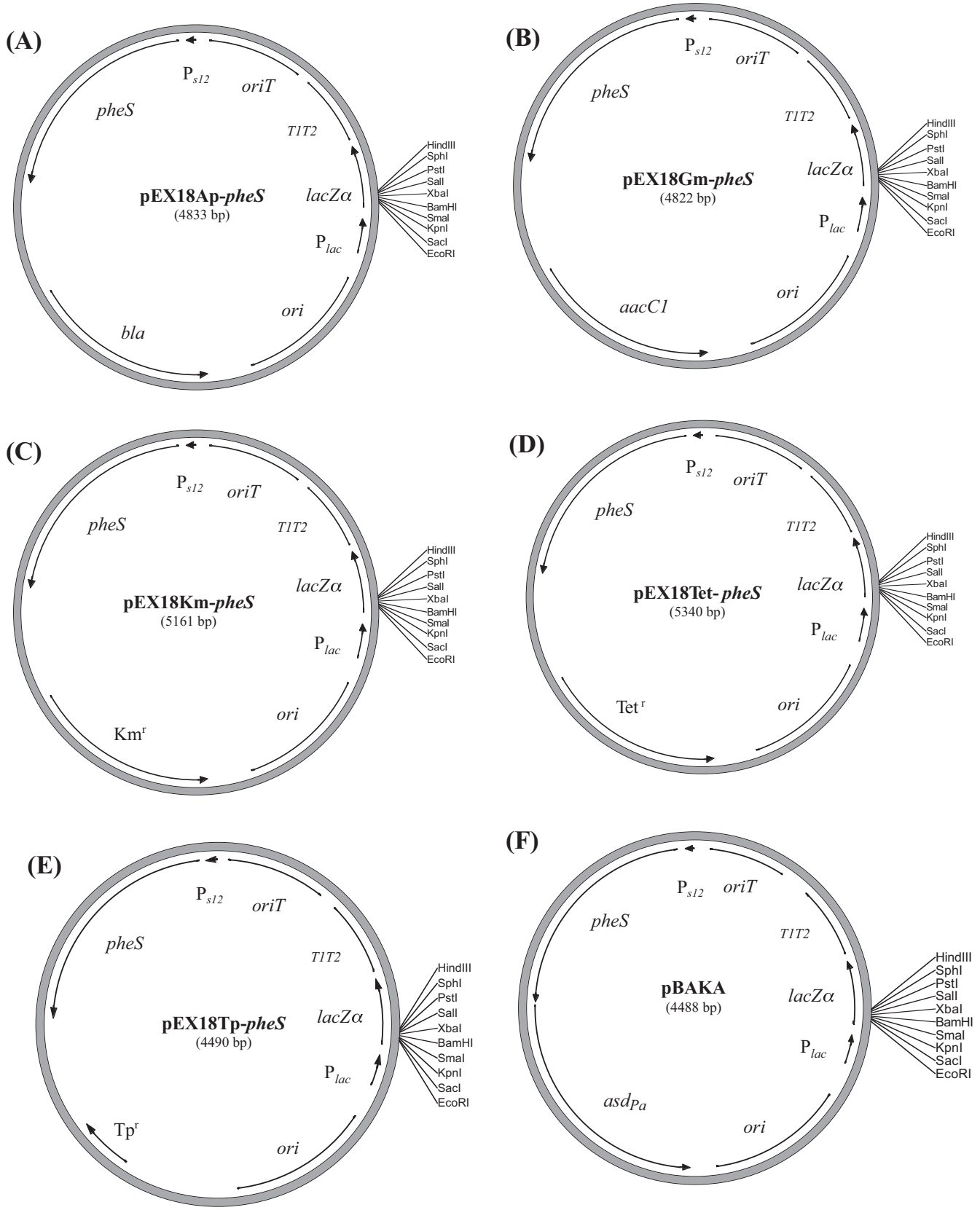

FIG. 2. Allelic replacement vectors based on the mutant *pheS* gene. Each vector contains a different selectable marker for resistance to Ap (A), Gm (B), Km (C), Tet (D), and Tp (E). (F) The asd_{Pa} gene as a non-antibiotic-selectable marker. All plasmids can be maintained in regular laboratory *E. coli* strains, with the exception of pBAKA, which was maintained in *E. coli* strain E1345 or E1354 (Table 1). *aacC1*, Gm acetyltransferase-encoding gene; *bla*, β-lactamase-encoding gene; *lacZ*α, β-galactosidase α-peptide; *ori*, ColE1 origin of replication; *oriT*, conjugal origin of transfer; P_{lac}, *lac* promoter; P_{S12}, the *B. pseudomallei rpsL* gene promoter; asd_{Pa}, *P. aeruginosa* aspartate semialdehyde dehydrogenase gene; *pheS*, mutant gene for the α-subunit of phenylalanyl tRNA synthase; and *T1T2*, transcriptional terminators.

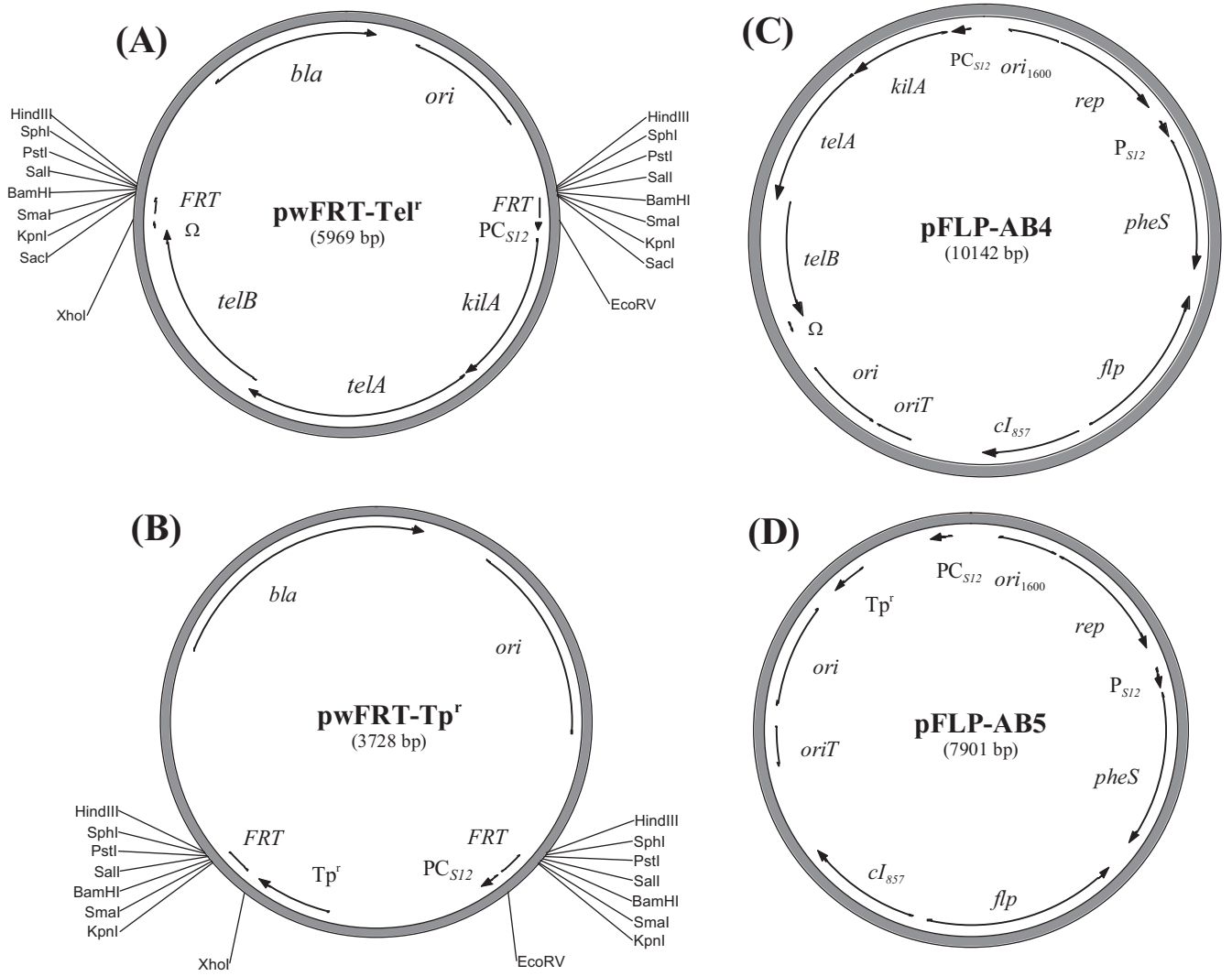

FIG. 3. Maps of FRT and Flp plasmids. Tel^r and Tp^r FRT cassettes can be removed by restriction digestion from pwFRT-Tel^r (A) and pwFRT-Tp^r (B), respectively. Not shown are four other unique FRT cassettes for each resistance determinant, which yield eight other plasmids (pmFRT-Tel^r, pmFRT-Tp^r, pFRT1-Tel^r, pFRT1-Tp^r, pFRT2-Tel^r, pFRT2-Tp^r, pFRT3-Tel^r, and pFRT3-Tp^r), where each selectable marker is flanked by identical FRTs with unique spacer sequences. The DNA sequences and restriction sites for all five Tel^r-FRT plasmids are identical, with the exception of the FRT spacer sequences on both sides of the resistant marker; similarly, all Tp^r-FRT plasmids have identical DNA sequences, with the exception of the spacers. Two Flp-containing replicative plasmids, pFLP-AB4 (C) and pFLP-AB5 (D), were engineered to excise chromosomal markers based on Tel^r and Tp^r, respectively. *cI*$_{857}$, temperature-sensitive λ *cI* repressor; *flp*, gene encoding flippase (Flp); Ω, *tonB* transcriptional terminator; *ori*$_{1600}$-*rep*, broad-host-range replicon; P$_{S12}$, promoter of the *B. pseudomallei rpsL* gene; PC$_{S12}$, promoter of the *B. cenocepacia rpsL* gene.

ing of potential mutants on MPG and MPC yielded a 100% choline-auxotrophic phenotype. Further screening by PCR with oligonucleotides 867 and 868 showed that all were mutants (data not shown), and one of these mutants is shown in Fig. 4B.

As a minor note, the concentration of Tel used is cell density dependent in our experience. Typically, a higher concentration of Tel (125 μg/ml) was used to select for merodiploids or pFLP plasmids from the conjugation mix, in which cell densities are high. However, the purification of the resulting merodiploids or colonies harboring replicative pFLP plasmids must be done at a lower Tel (75 μg/ml) concentration. This lower Tel concentration should also be used in the counter selection on 0.1% cPhe to obtain double-crossover mutants and the curing of pFLP plasmids on plates. We also recommend this lower concentration of Tel (75 μg/ml) for cultures grown in liquid medium from single colonies.

Flp excision of chromosomal resistance markers. We successfully performed Flp excision on the chromosomal Tel^r cassette in the *B. thailandensis* Δ*betBA*::Tel^r-FRT mutant (Fig. 4A). The introduction of pFLP-AB5 (Fig. 3D) into this mutant resulted in the excision of the Tel^r cassette by selection on Tp-300. The excision of the chromosomal Tel^r cassette was confirmed by PCR (Fig. 4B). The curing of Flp-containing plasmids (pFLP-AB5) was easily performed on 1× M9 minimal medium–20 mM glucose–0.1% cPhe. This strategy allows

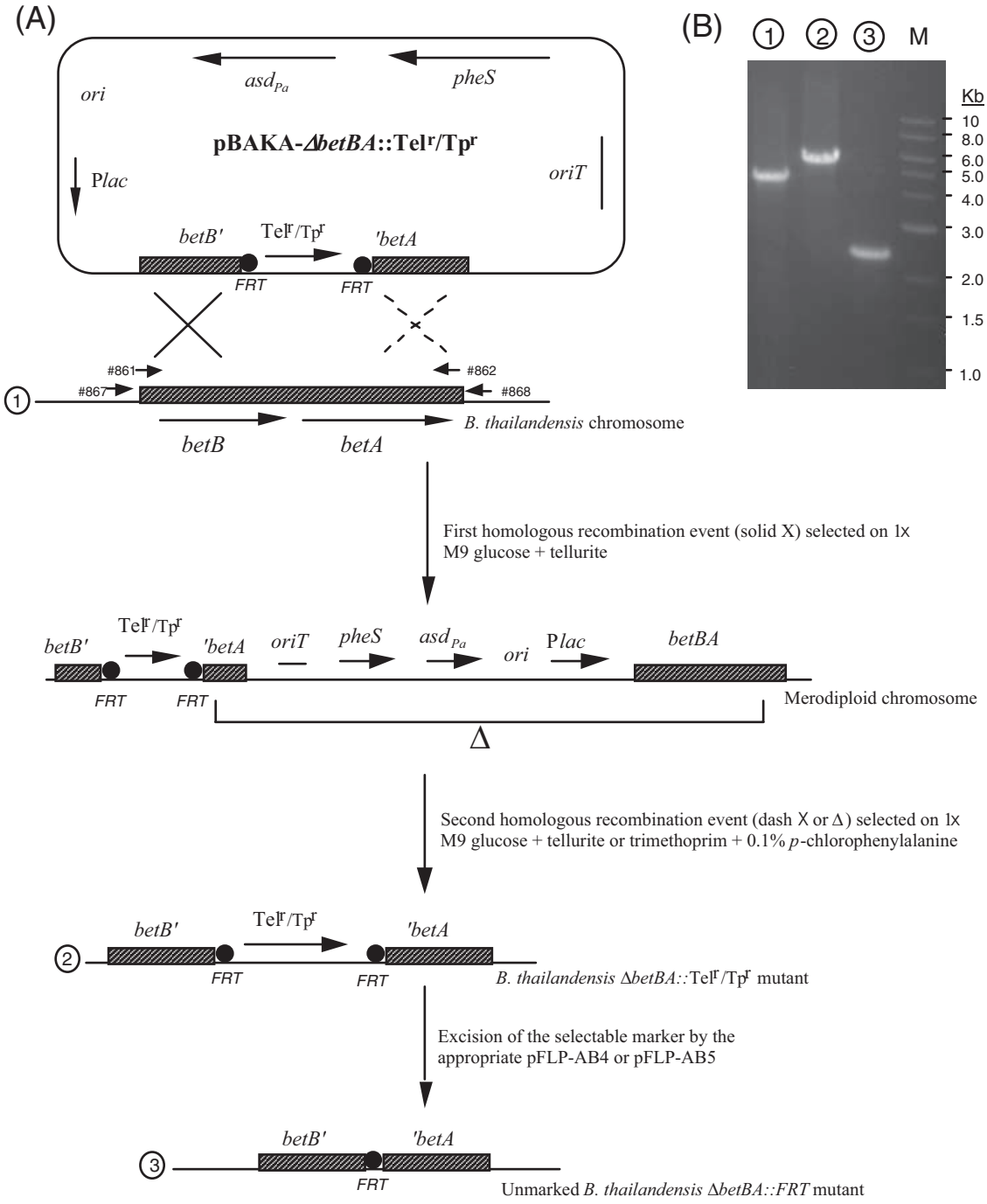

FIG. 4. (A) Gene replacement scheme using a Tel^r-FRT or Tp^r-FRT cassette to inactivate the *B. thailandensis betBA* operon. We utilized the Tel^r-FRT cassette. The *betBA* operon was amplified and cloned into pBAKA with oligonucleotides 861 and 862, and the inactivation and selection procedure performed was described in Materials and Methods. After the Flp excision, the resulting Δ*betBA*::FRT mutant has one remaining FRT sequence (~100 bp) that inactivates the *betBA* operon. (B) PCR confirmation of the Δ*betBA* mutant with outside oligonucleotides 867 and 868. Numbers in circles from 1 to 3 corresponds to lanes 1 to 3. Lane 1, wild-type *betBA* operon; lane 2, Δ*betBA*::Tel^r-FRT mutant before Flp excision; lane 3, Δ*betBA*::FRT mutant after Flp excision; M, 1-kb ladder.

the recycling and reuse of the same resistance marker for another round of allelic replacement. If the wFRT cassette is used in the first round of gene replacement, then the resistance marker in the second round of allelic replacement should be flanked identically by other FRTs with different spacer sequences (e.g., mFRT, FRT1, FRT2, or FRT3) (Fig. 3). This prevents undesirable chromosomal fragment deletions and rearrangements (1), as mentioned above. In our experience, the native Flp on pFLP-AB4 and pFLP-AB5 yielded an 80 to 100% efficiency of excision.

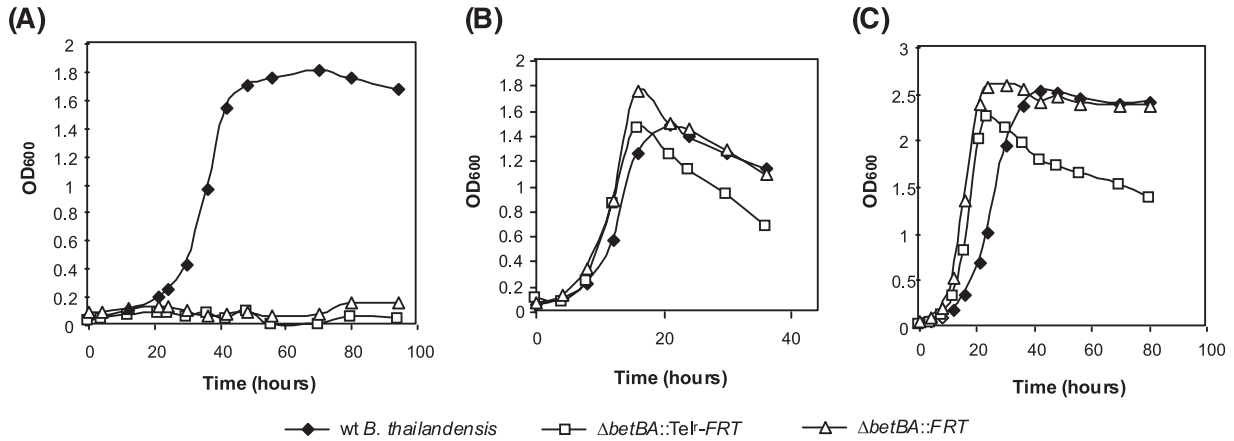

FIG. 5. Growth defect of the Δ*betBA*::Tel^r-FRT and Δ*betBA*::FRT mutants in choline, succinate, and glucose media. (A) The Δ*betBA* mutation abolished the growth of *B. thailandensis* in 1× M9 minimal medium–choline, while the wild type grew well on choline as a sole carbon source. The Δ*betBA*::FRT nonpolar mutation does not affect growth on the other carbon sources, succinate (B) and glucose (C), compared to that of wild-type *B. thailandensis*. However, quicker death was observed for the Δ*betBA*::Tel^r-FRT polar mutant (B and C).

***B. thailandensis* Δ*betBA* mutants are defective in choline degradation.** To further characterize the *B. thailandensis* Δ*betBA*::Tel^r-FRT and the Flp-excised Δ*betBA*::FRT mutants, we performed growth curve experiments for these mutants and compared the results to those for wild-type *B. thailandensis*. The results indicated that the *betBA* operon is involved in choline degradation (Fig. 5A). There are no other betaine aldehyde dehydrogenase (BetB) and choline dehydrogenase (BetA) homologs in *B. thailandensis*, because the Δ*betBA*::Tel^r-FRT and Δ*betBA*::FRT mutants were unable to grow on choline as a sole carbon source. The engineered Δ*betBA*::FRT mutation affected choline degradation and not the degradation of other carbon sources, such as succinate and glucose (Fig. 5). However, when grown in succinate and glucose media, death occurred more quickly with the Δ*betBA*::Tel^r-FRT mutant than with wild-type *B. thailandensis* and the Flp-excised Δ*betBA*::FRT mutant. We reasoned that there is a polar effect in the Δ*betBA*::Tel^r-FRT mutant due to a *tonB* transcriptional terminator in the Tel^r-FRT cassette that was constructed and used for allelic replacement (Fig. 3A). This polar effect was eliminated after the Flp excision of the *tonB* transcriptional terminator and the Tel^r cassette (Fig. 5). The ability to easily create a polar mutation and a derivative nonpolar mutation by using this Flp-FRT system may be beneficial to study the polar effects of different genetic loci. The regulation mechanism of the *betBA* operon is currently unknown. The future development of genetic tools is required to study the regulation of this *betBA* operon in *B. thailandensis*.

Conclusions. (i) We have engineered a broad-host-range counter-selectable marker, *pheS*, for *Burkholderia* species. (ii) Several allelic replacement vectors were constructed that were based on the mutant *pheS* gene, which will aid in gene replacement. Specifically, for *B. mallei* and *B. pseudomallei*, pBAKA along with *E. coli* cloning and delivery strains (E1345 and E1354) should be useful nonrestricted tools for allelic replacement. (iii) Ten unique FRT cassettes, based on two different resistance markers, allow repetitive rounds of allelic replacement that use the same selectable marker. The five Tel^r-FRT nonantibiotic cassettes will be particularly useful for *B. mallei* and *B. pseudomallei* studies. Although the effective selectable concentration has to be determined, it has been previously shown that *B. pseudomallei* is very sensitive to Tel (<1 μg/ml) (M. Frazier, K. Choi, A. Kumar, C. Lopez, R. R. Karkhoff-Schweizer, and H. P. Schweizer, 2007 American Society for Microbiology Biodefense and Emerging Diseases Research Meeting, Washington, DC). (iv) Two Flp-containing plasmids were engineered with the mutant *pheS* for self curing after the chromosomal excision of useful selectable markers. For *B. mallei* and *B. pseudomallei*, we recommend the combination of pBAKA and Tel^r-FRT for allelic replacement, because the whole process excludes the use of any clinically important antibiotics and antibiotic resistance markers. The Flp excision of the chromosomal Tel^r-FRT cassette could be performed with the select agent-compliant Flp plasmids pFLPe2 and pFLPe4, which were recently described (7). (v) These genetic systems were used to mutate the *B. thailandensis betBA* operon, demonstrating the function of BetB and BetA in choline degradation.

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