APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2009, p. 6062–6075 Vol. 75, No. 19
0099-2240/09/$08.00+0 doi:10.1128/AEM.00820-09
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Glyphosate Resistance as a Novel Select-Agent-Compliant, Non-Antibiotic-Selectable Marker in Chromosomal Mutagenesis of the Essential Genes *asd* and *dapB* of *Burkholderia pseudomallei*[▽]

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Received 5 April 2009/Accepted 26 July 2009

Genetic manipulation of the category B select agents *Burkholderia pseudomallei* and *Burkholderia mallei* has been stifled due to the lack of compliant selectable markers. Hence, there is a need for additional select-agent-compliant selectable markers. We engineered a selectable marker based on the *gat* gene (encoding glyphosate acetyltransferase), which confers resistance to the common herbicide glyphosate (GS). To show the ability of GS to inhibit bacterial growth, we determined the effective concentrations of GS against *Escherichia coli* and several *Burkholderia* species. Plasmids based on *gat*, flanked by unique flip recombination target (*FRT*) sequences, were constructed for allelic-replacement. Both allelic-replacement approaches, one using the counterselectable marker *pheS* and the *gat-FRT* cassette and one using the DNA incubation method with the *gat-FRT* cassette, were successfully utilized to create deletions in the *asd* and *dapB* genes of wild-type *B. pseudomallei* strains. The *asd* and *dapB* genes encode an aspartate-semialdehyde dehydrogenase (BPSS1704, chromosome 2) and dihydrodipicolinate reductase (BPSL2941, chromosome 1), respectively. Mutants unable to grow on media without diaminopimelate (DAP) and other amino acids of this pathway were PCR verified. These mutants displayed cellular morphologies consistent with the inability to cross-link peptidoglycan in the absence of DAP. The *B. pseudomallei* 1026b Δ*asd*::*gat-FRT* mutant was complemented with the *B. pseudomallei asd* gene on a site-specific transposon, mini-Tn*7-bar*, by selecting for the *bar* gene (encoding bialaphos/PPT resistance) with PPT. We conclude that the *gat* gene is one of very few appropriate, effective, and beneficial compliant markers available for *Burkholderia* select-agent species. Together with the *bar* gene, the *gat* cassette will facilitate various genetic manipulations of *Burkholderia* select-agent species.

Members of the genus *Burkholderia*, comprising more than 40 different species, are extremely diverse gram-negative, nonspore-forming bacilli. Many *Burkholderia* species exist as innocuous soil saprophytes or plant pathogens (47), while others cause human and animal diseases. Among these human and animal pathogens are the etiological agents of melioidosis (*Burkholderia pseudomallei*) and glanders (*Burkholderia mallei*) (9, 50, 51). Melioidosis is an emerging infectious disease generally considered endemic to Southeast Asia and Northern Australia (12). Positive diagnoses in many tropical countries around the world have expanded the global awareness of melioidosis (3, 15, 24, 25, 28, 35, 39, 42, 52). In contrast to the ubiquitous nature of *B. pseudomallei*, *B. mallei* is also a highly infectious agent causing glanders, a predominantly equine disease (34, 50). *B. mallei*, a clone derived from genomic downsizing of *B. pseudomallei*, has been used in biowarfare (17). This historical significance, along with the low infectious dose and the route of infection, has contributed to the decision by the Centers for Disease Control and Prevention (CDC) to classify these two microbes as category B select agents (43).

Classification of *B. pseudomallei* as a select agent has stimulated interest and research into the pathogenesis of melioidosis, necessitating the development of appropriate tools for genetic manipulation. In the struggle to elucidate the molecular mechanisms of pathogenesis, selectable markers are indispensable genetic tools (45). Current CDC regulations prohibit the cloning of clinically important antibiotic resistance genes into human, animal, or plant select-agent pathogens if the transfer could compromise the ability to treat or control the disease. The only antibiotic markers currently approved for use in *B. pseudomallei* are based on resistance to aminoglycosides (gentamicin, kanamycin, and zeocin) (45). However, the efficacy of these markers is limited, due to high levels of aminoglycoside resistance inherent within the *Burkholderia* genus and high levels of spontaneous aminoglycoside resistance in *B. pseudomallei* (10, 19, 41). In addition, the use of aminoglycosides (e.g., gentamicin) for selection may require aminoglycoside efflux pump mutants (10, 33). Another potential drawback is that efflux pumps play a major role in bacterial physiology, and mutating them may change the pathogenic traits under investigation (7, 40). A more logical approach employs alternative, non-antibiotic-selectable markers conferring resistance to compounds that are not potentially important in clinical treatment.

Very few non-antibiotic resistance markers have been utilized successfully for *Burkholderia* species. A non-antibiotic-selectable-marker based on tellurite resistance (Tel^r) has been successfully developed and used with *Pseudomonas putida*,

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▽ Published ahead of print on 31 July 2009.

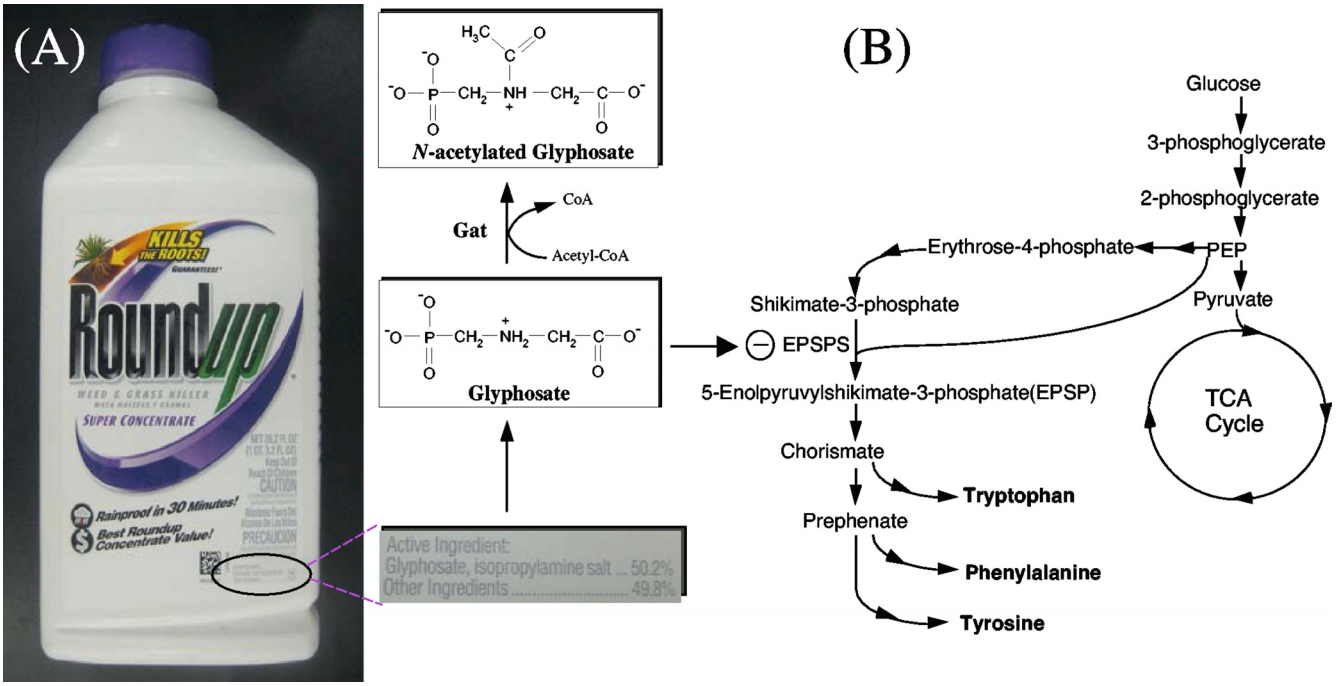

FIG. 1. (A) A 946-ml bottle of the "superconcentrated" herbicide Roundup used in this study, available for ~$50 from most local hardware stores and garden or farm supply centers. The active ingredient, 50% GS, is indicated on the label, and the chemical structure of GS is shown. GAT, encoded by the *gat* gene, catalyzes the inactivation of GS via N acetylation. (B) Pathways of aromatic amino acid biosynthesis. GS inhibits the enzyme EPSPS, which is required for the biosynthesis of aromatic amino acids, thus starving bacteria for tyrosine, phenylalanine, and tryptophan. PEP, phosphoenolpyruvate; TCA cycle, tricarboxylic acid cycle.

Pseudomonas fluorescens, and *Burkholderia thailandensis* (2, 27, 44). The engineering of Tel^r-*FRT* (flip recombination target) cassettes, coupled to *FRT* sequences, could be used to generate unmarked mutations and allow recycling of the Tel^r selectable-marker (2). In addition, utilization of Flp-*FRT* resistance cassettes to generate mutants allows downstream modification and manipulation such as fusion integration (29). However, the disadvantage of the Tel^r-cassette is the number of genes required (*kilA-telA-telB*) and the large size (>3 kb), making it less likely to obtain PCR products for allelic replacement by natural transformation (46). Another potentially useful non-antibiotic-selectable marker is based on the *bar* gene, encoding resistance to bialaphos or its degradation product, phosphinothricin (PPT) (49). PPT inhibits glutamine synthetase in plants (48), starving the cell for glutamine, and the *bar* gene has been used successfully as a selection marker in gram-negative bacteria (21). For select-agent *Burkholderia* species, however, the PPT MIC was found to be greater than 1,024 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). We have found the effective concentration of PPT for *B. pseudomallei* and *B. mallei* to be ~2.5% (25,000 μg/ml [data not shown]). The high concentration of PPT required for selection in these species may be costly, considering that purified PPT costs ~$380 per g. Therefore, further development of non-antibiotic resistance markers, as well as a more economical source of herbicide for use with restricted select-agent species, is needed.

Work by Castle et al. (5) generated a highly active glyphosate *N*-acetyltransferase (GAT) enzyme for plant engineering, making it possible to utilize the *gat* gene as an effective non-antibiotic resistance marker for bacterial selection with glyphosate (GS). The commonly used herbicide GS inhibits the 5-enolpyruvylshikimate-3-phospate synthase (EPSPS) of plants through competition with phosphoenolpyruvate for overlapping binding sites on EPSPS (14), depriving plants of three aromatic amino acids (Fig. 1). Since humans and animals obtain tryptophan and phenylalanine (giving rise to tyrosine) through dietary intake, GS is relatively nontoxic. Like plants, bacteria must make these amino acids, when they are lacking, from basic precursors. GS has been found to be inhibitory to a variety of bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Bradyrhizobium japonicum* (16, 55), while other bacterial strains are able to metabolize low concentrations of GS (26, 31). Although *B. pseudomallei* has been reported to have two genes (*glpA* and *glpB*) for GS degradation and metabolism (38), our searches of all available genomes of *Burkholderia* species in GenBank yielded no *glpA* or *glpB* genes within this genus. GS resistance by bacteria has been documented through EPSPS target mutations or GS detoxification mechanisms (36). However, these mechanisms did not confer resistance to relatively high GS concentrations. More recently, directed evolution of the *gat* gene, based on various bacterial *gat* sequences and selection in *E. coli*, yielded a very active GAT protein sequence with an efficiency increase of nearly 4 orders of magnitude (5), holding promise as an

TABLE 1. Bacterial strains used in this study[a]

Strain	Lab ID[b]	Relevant properties	Source
E. coli			
K-12	E0577	Wild type; F^+	Coli Genetic Stock Center
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-*wFRT*	—[c]
EPMax10B-*lacI*^{q}/*pir*/*leu*$^+$/Δ*asd*::Gm^r	E1951	Gm^r; F^- λ^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 galU galK rpsL nupG lacI*^{q}*-FRT8 pir::FRT4* Δ*asd*::Gm^r-*wFRT*	—[c]
EPMax10B-*pir116*/Δ*asd*/Δ*trp*::Gm^r/ *mob*-Km^r	E1354	Gm^r Km^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*::*wFRT* Δ*trp*::Gm^r-*FRT5 mob*[*recA*::*RP4-2 Tc::Mu-Km*^{r}]	—[c]
EPMax10B-*lacI*^{q}/*pir*	E1869	F^- λ^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ*(ara leu)7697 galU galK rpsL nupG lacI*^{q}*-FRT8 pir-FRT4*	—[c]
EPMax10B-*lacI*^{q}/*pir*/*leu*$^+$	E1889	F^- λ^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 galU galK rpsL nupG lacI*^{q}*-FRT8 pir-FRT4*	—[c]
GM33	E0021	F^- λ^- IN(*rrnD-rrnE*)*1 dam-3 sup-85*	32
Burkholderia spp.			
B. dolosa AU0158	E1551	Prototroph	J. Goldberg
B. cenocepacia			
Bc7	E1552	Prototroph	J. Goldberg
K56-2	E1554	Prototroph; cystic fibrosis isolate	P. Sokol
B. mallei ATCC 23344	B0002	Wild-type strain; postmortem isolate	34
B. pseudomallei			
1026b	B0004	Wild-type strain; clinical melioidosis isolate	13
1026b-Δasd_{Bp}::*gat-FRT*	B0011	GS^r; 1026b with a *gat-FRT* cassette inserted into the asd_{Bp} gene	This study
1026b-Δasd_{Bp}::*gat-FRT*/*att*Tn*7-bar-*asd_{Bp}	B0015	GS^r PPT^r; 1026b Δasd_{Bp}::*gat-FRT* mutant with mini-Tn*7-bar-*asd_{Bp} inserted	This study
1026b-Δ$dapB_{Bp}$::*gat-FRT*	B0013	GS^r; 1026b with a *gat* cassette inserted into the $dapB_{Bp}$ gene	This study
K96243	B0002	Wild-type strain; clinical melioidosis isolate	23
K96243-Δasd_{Bp}::*gat-FRT*	B0007	GS^r; K96243 with a *gat* cassette inserted into the asd_{Bp} gene	This study
K96243-Δ$dapB_{Bp}$::*gat-FRT*	B0010	GS^r; K96243 with a *gat* cassette inserted into the chromosomal $dapB_{Bp}$ gene	This study
B. thailandensis E264	E1298	Prototroph; environmental isolate	4

[a] Abbreviations and designations: *bar*, gene encoding bialaphos (PPT) resistance; *gat*, gene encoding GAT; Gm^r, gentamicin resistant; GS^r, glyphosate resistant; Km^r, kanamycin resistant; PPT^r, PPT resistant.
[b] Please use the laboratory identification number (lab ID) when requesting strains.
[c] Details on the engineering of this strain are to be published elsewhere.

appropriate non-antibiotic resistance marker for select-agent species.

Here we engineered and tested a novel non-antibiotic-selectable-marker (*gat*) for use in the select agent *B. pseudomallei*. GS is the active ingredient in Roundup, which was used for selection (Fig. 1). The effective compound GS is readily available, inexpensive, relatively nontoxic, very soluble, and not clinically important, and it yields tight selection. The engineered *gat* marker (563 bp) was optimized for *Burkholderia* codon usage and adapted (with a *Burkholderia rpsL* promoter) for use in the select agent *B. pseudomallei*. Effective concentrations of GS for several species of *Burkholderia*, including the select agents *B. pseudomallei* and *B. mallei*, were determined. Using the *gat* gene, we created deletion mutants of the essential *B. pseudomallei asd* and *B. pseudomallei dapB* (asd_{Bp} and $dapB_{Bp}$) genes (encoding aspartate-semialdehyde dehydrogenase and dihydrodipicolinate reductase, respectively) in two wild-type *B. pseudomallei* strains. The Δasd_{Bp} mutant of *B. pseudomallei* showed a phenotypic defect consistent with the lack of diaminopimelate (DAP) for cell wall cross-linking. Complementation of the *B. pseudomallei* Δasd_{Bp} mutant with the asd_{Bp} gene located on a site-specific transposon, mini-Tn*7-bar*, was successful by using an inexpensive source of PPT for selection.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. All strains and plasmids used in this study are listed in Tables 1 and 2. All manipulations with *B.*

TABLE 2. Plasmids used in this study[a]

Plasmid	Lab ID[b]	Relevant properties	Source
mini-Tn*7*-Tel^r	E1825	Tel^r; mini-Tn*7* integration vector based on Tel^r	29
mini-Tn*7*-*bar*	E2218	PPT^r; mini-Tn*7* integration vector based on *bar*	This study
mini-Tn*7*-*gat*	E1981	GS^r; mini-Tn*7* integration vector based on *gat*	This study
mini-Tn*7*-*bar*-*asd*$_{Bp}$	E2226	PPT^r; *B. pseudomallei* K96243 *asd*$_{Bp}$ gene cloned into mini-Tn*7*-*bar*	This study
pBAKA	E1624	Select-agent-compliant allelic-replacement vector based on *asd*$_{Pa}$	2
pBAKA-Δ*asd*$_{Bp}$::*gat-FRT*	E2062	GS^r; *gat-FRT* cassette inserted into *asd*$_{Bp}$	This study
pBAKA-*dapB*$_{Bp}$	E2075	*B. pseudomallei* K96243 *dapB* gene cloned into pBAKA	This study
pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*	E2083	GS^r; *gat-FRT* cassette inserted into *dapB*$_{Bp}$	This study
pBBR1MCS-2	E1277	Km^r; broad-host-range cloning vector	30
pBBR1MCS-2-PC$_{S12}$-*bar*	E1773	Km^r PPT^r; broad-host-range cloning vector harboring *bar*	This study
pBBR1MCS-2-PC$_{S12}$-*gat*	E1794	GS^r Km^r; broad-host-range cloning vector harboring *gat*	This study
pCAMBIA-1301-*bar*	E1775	PPT^r; plant transformation vector harboring *bar*	Cambia
pTNS3-*asd*$_{Ec}$	E1831	Helper plasmid containing *asd*$_{Ec}$ for Tn*7* site-specific transposition system	29
pUC18	E0135	Ap^r; cloning vector	53
pUC18-*asd*$_{Bp}$	E1819	Ap^r; cloning vector pUC18 containing *asd*$_{Bp}$	This study
pUC18-Δ*asd*$_{Bp}$::*gat-FRT*	E1867	Ap^r GS^r; cloning vector pUC18 containing *asd*$_{Bp}$ inactivated by *gat-FRT*	This study
pUC57-P$_{S12}$-*gat*	E1763	Ap^r GS^r; cloning vector pUC57 containing *B. pseudomallei* codon-optimized *gat*	This study
pwFRT-P$_{S12}$-*gat*	E1798	Ap^r GS^r; *gat* cassette flanked by wild-type *FRT*	This study
pwFRT-P$_{S12}$-*gat*-SDM	E1812	Ap^r GS^r; *gat* cassette flanked by wild-type *FRT* after SDM removing an internal SacI site	This study
pwFRT-PC$_{S12}$-*gat*[c]	E1929	Ap^r GS^r; *gat* cassette flanked by wild-type *FRT* with the P$_{S12}$ replaced by the PC$_{S12}$	This study
pwFRT-PC$_{S12}$-*bar*	E2209	Ap^r PPT^r; *bar* cassette flanked by wild-type *FRT*	This study
pwFRT-Tp^r	E1659	Tp^r; Tp^r cassette flanked by wild-type *FRT*	2
pwFRT-Tel^r	E1584	Tel^r; Tel^r cassette flanked by wild-type *FRT*	2

[a] Ap^r, ampicillin resistant; PC$_{S12}$, *rpsL* promoter of *B. cenocepacia*; P$_{S12}$, *rpsL* promoter of *B. pseudomallei.*
[b] Please use the laboratory identification number (Lab ID) when requesting plasmids.
[c] Four other *FRT* mutants exist for this plasmid, where the only sequence difference in the mutated plasmids is within the spacer sequence of each *FRT* (see Materials and Methods for details).

pseudomallei and *B. mallei* were conducted in a CDC/USDA-approved and -registered BSL3 facility at the University of Hawaii at Manoa, and experiments with these select agents were performed with BSL3 practices by following the recommendations of *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition (51a).

Luria-Bertani (LB) medium (Difco) was used to culture all *E. coli* strains. *Burkholderia* strains (*B. pseudomallei*, *B. mallei*, *B. thailandensis*, *Burkholderia cenocepacia*, and *Burkholderia dolosa*) were cultured in LB medium or 1× M9 minimal medium plus 20 mM glucose (MG medium). DAP was prepared in 1 M NaOH as a 100-mg/ml stock and was used when necessary as described previously (2). A ~1-liter bottle of the "superconcentrated" herbicide Roundup (50% [wt/vol] GS) was purchased at a City Mill hardware store as a source of GS for approximately $50 and was used in this study. Purified GS was purchased from Sigma. We also purchased the herbicide Finale (9.5 liters with 11.33% [wt/vol] PPT) for $125 at a local farm supply store (Pacific Agricultural Sales and Services), and it was used as a source of PPT in this study. MG medium plus GS or PPT was utilized for *gat* or *bar* selection, respectively. Since GS blocks the biosynthesis of aromatic amino acids (Fig. 1), it is necessary to use a minimal medium without Phe, Trp, or Tyr (e.g., MG medium). We observed that minimal medium provided with any two aromatic amino acids abolished the selective potential of GS. Likewise, minimal medium lacking glutamine is required for the selection of *bar* with PPT. Antibiotics and nonantibiotic antibacterial compounds in solid media were utilized as follows: for *E. coli*, ampicillin at 110 μg/ml, 0.3% GS, kanamycin (Km) at 35 μg/ml, and 0.3% PPT; for *B. mallei*, 0.2% GS (effective concentration); for *B. pseudomallei*, 0.3% GS and 2.5% PPT; and for *B. thailandensis*, Km at 500 μg/ml, 0.04% GS, and 1.5% PPT.

Two derivatives of *E. coli* EPmax10B (Bio-Rad), one containing *lacI*^{q} and *pir* (laboratory identification no. E1869) and the other containing *lacI*^{q}, *pir*, and *leu*$^+$ (E1889), were routinely used as cloning strains in rich and minimal media, respectively. The *E. coli* conjugal and suicidal strain EPMax10B-*pir116*-Δ*asd*-*mob*-Km-Δ*trp*::Gm (E1354) was used for plasmid mobilization into *B. pseudomallei* and *B. thailandensis*. Growth of *E. coli* Δ*asd* strains was carried out as previously described (2). *E. coli* strain EPMax10B-*pir116*-Δ*asd*::Gm (E1345) was used for the cloning of *asd*-complementing vectors (e.g., pBAKA) (the *asd* gene encodes aspartate-semialdehyde dehydrogenase). Briefly, selection of E1345 complemented with various *asd*- and *gat*-containing constructs (e.g., pBAKA-Δ*asd*$_{Bp}$::*gat-FRT* or pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*) was performed on MG-plus-GS medium supplemented with leucine (Leu). To simplify selection and replace strain E1345, EPMax10B-*lacI*^{q}/*pir*/*leu*$^+$/Δ*asd*::Gm (E1951) was later created to select for *asd*-, *bar*-, and *gat*-containing plasmids on MG-plus-PPT or MG-plus-GS medium, so that leucine (Leu) could be omitted from the minimal medium. Selection of *asd*-, *bar*-, and *gat*-containing plasmids in the conjugation-proficient strain E1354 was carried out with MG medium plus Leu, Trp, and GS or PPT; in the absence of a complementing *asd* gene (e.g., pBBR1MCS-2-PC$_{S12}$-*gat*), an additional 1 mM (each) lysine (Lys), methionine (Met),and threonine (Thr) and 100 μg/ml of DAP were added. For selection against E1354 following conjugation, Leu and Trp were omitted from the growth medium. Counterselection of *pheS* was carried out on MG medium containing 0.1% *p*-chlorophenylalanine (cPhe; DL-4-chlorophenylalanine from Acros Organics) as described previously (2). *B. pseudomallei* Δ*asd*$_{Bp}$::*gat-FRT* and Δ*dapB*$_{Bp}$::*gat-FRT* mutants were grown on rich LB medium plus 200 μg/ml DAP. For Δ*dapB*$_{Bp}$::*gat-FRT* mutants grown in minimal medium, MG medium plus 200 μg/ml DAP and 1 mM Lys was used; this minimal medium was also supplemented with 1 mM of both Met and Thr for growing Δ*asd*$_{Bp}$::*gat-FRT* mutants.

Molecular methods and reagents. The oligonucleotides used in this study are listed in Table 3. All molecular methods and reagents used have been described previously (2).

Conjugation into *Burkholderia* spp. Conjugation between the *E. coli* strain E1354 and *Burkholderia* strains was routinely carried out as described previously (29) with the modifications described below. After conjugation, cells were resuspended and washed twice in 1 ml of 1× M9 buffer (to remove trace amino acids) and then resuspended in 1 ml of 1× M9 buffer; 100-μl and 200-μl aliquots of the cell suspensions were plated onto the appropriate media. Conjugation using this method usually resulted in 50 to 100 colonies for recombination of nonreplicating vectors when 100 μl of a 1-ml conjugation recovery culture was plated and 500 to 700 colonies for replicating plasmids when 100 μl of a 10× dilution was plated.

Growth inhibition of *Burkholderia* select-agent species in GS-containing medium after 24 h. We wanted to determine if GS had a growth-inhibiting and killing effect by exposing *B. pseudomallei* strains 1026b and K96243 and *B. mallei* ATCC 23344 to increasing concentrations of GS for 24 h in minimal glucose medium. First, *B. mallei* and the two *B. pseudomallei* strains (1026b and K96243) were grown overnight in LB medium. The cultures were washed twice in 1 ml of 1× M9 buffer, resuspended in 1 ml of the same buffer, and used to inoculate

TABLE 3. Oligonucleotide primers used in this study

Primer no.	Primer name	Sequence[a]
557	M13-RP	5′-AGCGGATAACAATTTCACACAGGA-3′
558	M13-FP	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
715	pPS854-XhoI	5′-AAGCTCGAGCTAATTCC-3′
716	pPS854-Cla-EcoRV	5′-CAATATCGATATCCATTGCTGTTGACAAAG-3′
837	PS12(cenocepacia)	5′-ATCAGCCGTTGACTTAGTTGGTATTTCCGGAATATCATGCTGGTTCCGAATAA TTTTGTTTAACTTTAAGAAGGAGATATACC-3′
849	Tel-term-BamHI	5′-TCGAGGATCCAGAAAGTCAAAAGCCTCCG-3′
876	Tn7L	5′-ATTAGCTTACGACGCTACACCC-3′
881	bar-start	5′CTTTAAGAAGGAGATATACCATGAGCCCAGAACGACGCC-3′
882	bar-XhoI	5′-GAAACTCGAGTCAAATCTCGGTGCCGGGCA-3′
892	Bpasdup-HindIII	5′-CGTCAAGCTTTCCCGGCCGTTGTG-3′
893	Bpasddown-EcoRI	5′-GTTGTGAATTCGTCGTAATCGCGTAG-3′
894	gat-SacSDM	5′-GCACTCGGAGCTTCAGGGGAAGAAGC-3′
1048	dapB-up-XbaI	5′-CGGCTCTAGAAGCCATGCAGGCGG-3′
1049	dapB-up-nest	5′-GAGCAGAACGACGCGAAC-3′
1050	dapB-down-HindIII	5′-CGAGAAGCTTGTACGCGAGCACCG-3′
1051	dapB-down-nest	5′-GAACGCGGTCATGATGAG-3′
1062	1026b-asd-up	5′-CCCGAAAACGGGGTCCGT-3′
1063	1026b-asd-dn	5′-CGACGCTTTCGGGTTGTGTA-3′
1070	dapB-dn-out	5′-CAGACGAACACGTGCAGATC-3′
1071	dapBK9-upout-2	5′-AGCTCGATCTGCTCGCCGACAT-3′
1079	glmS1-K9	5′-GAGGAGTGGGCGTCGATCAAC-3′
1080	glmS2-K9	5′-ACACGACGCAAGAGCGGAATC-3′
1081	glmS3-K9	5′-CGGACAGGTTCGCGCCATGC-3′
1117	BpK9asd-upstrm-HindIII	5′-GCGCGAAGCTTTCGACACGATG-3′

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

(1:100 dilution) into 3 ml of MG medium plus 0.25%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, or 4.0% GS with 30 μl of the washed cultures. Immediately, 100 μl of serial dilutions of each culture was plated to determine initial bacterial CFU/ml for each strain. After 24 h, 100 μl of serial dilutions from each culture was plated onto LB plates, and bacterial CFU/ml were again determined. We used the ratio of bacterial CFU/ml at 24 h to bacterial CFU/ml at the initial exposure to determine the percentage of survival after a 24-h exposure to GS (Fig. 2).

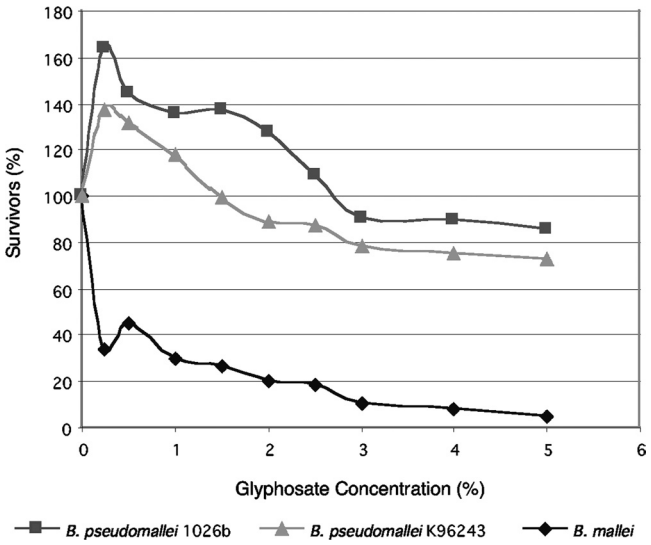

FIG. 2. Bacterial survival after incubation with different concentrations of GS for 24 h. *B. mallei* was more sensitive to GS than both *B. pseudomallei* strains, and killing of *B. mallei* by GS was observed at 0.25% GS. *B. pseudomallei* strain 1026b is significantly more resistant to GS than strain K96243. Minimal replication of both *B. pseudomallei* strains (less than doubling) after 24 h was observed at 0.25% GS. Killing was observed at 2% GS for strain K96243 and at 3% GS for strain 1026b.

Determination of GS MICs and GS_{EC}s. To determine the MIC of GS in liquid medium, we first grew all strains overnight in LB medium. One milliliter of culture was harvested by centrifugation, washed twice in 1× M9 buffer to remove trace amounts of amino acids, resuspended in 1× M9 buffer, and diluted 100× in the same buffer. GS gradients in MG medium, starting with a concentration of 0.8% GS and decreasing by 2× dilutions to 0.00625% GS, were inoculated with 100× dilutions (~10^5 CFU/ml) of each strain listed in Table 4. The MIC in liquid medium was then determined to be the concentration that showed no visible growth after 2 days of incubation, with shaking, at 37°C. To establish the MIC of GS on solid medium, we used MG medium plus GS and 1.5% (wt/vol) agar. Cultures of all species in LB medium were grown to an optical density at 600 nm of ~0.8. One milliliter of each culture was harvested as described above for MICs in liquid medium and was resuspended in 1× M9 buffer. One hundred microliters of the high-cell-density cultures was then plated onto MG medium plates containing different concentrations of GS (ranging from 0 to 0.5%). The concentration at which no growth was observed after 2 weeks was defined as the plate MIC. The GS concentration for each species was increased by ~30% above

TABLE 4. GS_{EC}s for *Burkholderia* species and *E. coli*

Strain	GS_{EC} (%) in:	
	Liquid medium[a]	Solid medium[b]
B. cenocepacia		
K56-2	0.32	0.5
Bc7	<0.005	<0.005
B. dolosa AUO158	<0.005	<0.005
B. mallei ATCC 23344	0.1	0.2
B. pseudomallei		
K96243	0.1	0.3
1026b	0.1	0.3
B. thailandensis E264	0.01	0.04
E. coli K-12	0.1	0.3

[a] Determined by the absence of growth after 4 days.
[b] Determined by the absence of growth or spontaneously resistant colonies after 3 weeks.

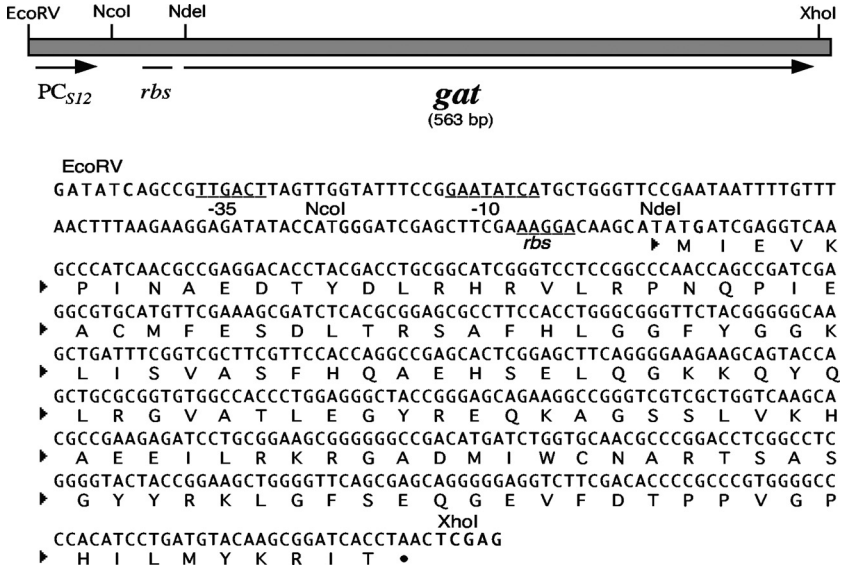

FIG. 3. Schematic diagram of the engineered 563-bp *gat* gene on pwFRT-PC$_{S12}$-*gat*. The *B. cenocepacia rpsL* promoter (PC$_{S12}$) and ribosomal binding site (*rbs*) are shown in relation to the *gat* gene. Below the schematic are the corresponding nucleotide and protein sequences. Codons were optimized according to the codon preference within the *B. pseudomallei* K96243 *asd* gene. Also indicated are the −35 and −10 regions of the PC$_{S12}$ promoter. Restriction sites (in boldface) were positioned strategically for subsequent cloning and manipulation.

the MIC, and complete growth inhibition after 4 days in liquid medium and 3 weeks on solid medium was empirically taken as the effective concentration of GS (GS$_{EC}$) (Table 4).

Engineering of pUC57-P$_{S12}$-*gat*, pwFRT-PC$_{S12}$-*gat*, and pBBR1MCS-2-PC$_{S12}$-*gat*. Driven by the *B. pseudomallei rpsL* promoter (P$_{S12}$) (54), the *gat* gene sequence was optimized to the codon usage of *B. pseudomallei*. The *gat* gene sequence was synthesized by GenScript Corporation and was cloned into pUC57 as an EcoRV-XhoI fragment, yielding pUC57-P$_{S12}$-*gat*. pUC57-P$_{S12}$-*gat* was first digested with EcoRV and XhoI and was then inserted into EcoRV- and XhoI-digested pwFRT-Tp^r, replacing the Tp^r-cassette and yielding pwFRT-P$_{S12}$-*gat*. We accidentally introduced a SacI restriction site during engineering, and it was removed by site-directed mutagenesis (SDM) using oligonucleotide 894 to yield pwFRT-P$_{S12}$-*gat*-SDM. Additionally, the *gat* gene was initially engineered to be driven by P$_{S12}$ on pUC57-P$_{S12}$-*gat*. However, homologous recombination may occur with the P$_{S12}$ region at the native locus on the chromosome or with an additional P$_{S12}$ located on pBAKA. To prevent this possibility, the *gat* gene from pwFRT-P$_{S12}$-*gat*-SDM was removed using NcoI and XhoI and was ligated into pwFRT-Tel^r cut with the same enzymes, replacing the Tel^r cassette with the *gat* gene, yielding pwFRT-PC$_{S12}$-*gat* (Fig. 3 and 4A). Unique enzyme sites are present on the *gat* cassette to allow for ease of manipulation (Fig. 4A).

pBBR1MCS-2-PC$_{S12}$-*gat* was constructed to test the effectiveness of GS selection in *E. coli* and *B. thailandensis* by comparing colony numbers on LB-plus-Km medium with those on MG-plus-GS medium. pBBR1MCS-2 was digested with KpnI, blunt ended, and then digested with XhoI. The resultant fragment was ligated to the 563-bp EcoRV-XhoI PC$_{S12}$-*gat* fragment from pwFRT-PC$_{S12}$-*gat*, yielding pBBR1MCS-2-PC$_{S12}$-*gat*.

Determination of the PPT$_{EC}$ and construction of pBBR1MCS-2-PC$_{S12}$-*bar*. The effective concentration of PPT (PPT$_{EC}$) from the herbicide Finale was determined in the same manner as the GS$_{EC}$. We first determined whether *bar* would be an efficient selectable marker in *E. coli* and *B. thailandensis* by constructing pBBR1MCS-2-PC$_{S12}$-*bar*. The *bar* gene was amplified from pCAMBIA-1301-*bar* using oligonucleotides 881 and 882. The product was then used as a template for a second PCR using oligonucleotides 837 and 882 to introduce the *B. cenocepacia rpsL* promoter (PC$_{S12}$). The 800-bp PCR product was then digested with XhoI and ligated into EcoRV- and XhoI-digested pBBR1MCS-2 to yield pBBR1MCS-2-PC$_{S12}$-*bar*. This construct was then introduced into *E. coli* and subsequently into *B. thailandensis* via electroporation. The PPT$_{EC}$s were determined to be 0.3% and 1.5% for *E. coli* and *B. thailandensis*, respectively. Introduction of pBBR1MCS-2-PC$_{S12}$-*bar* into *E. coli* and *B. thailandensis* yielded the same number of colonies on kanamycin-containing medium as on PPT-containing medium (data not shown), indicating that this source of PPT contains no other ingredients that could adversely affect the selection of *bar*-containing constructs. The PPT$_{EC}$s for *B. pseudomallei* 1026b, *B. pseudomallei* K96243, and *B. mallei* were found to be 2.5% by the methods described above for the GS$_{EC}$.

Construction of *gat-FRT* and *bar-FRT* vectors. Using pwFRT-PC$_{S12}$-*gat* with TCTAGAAA as the wild-type spacer of the flanking *FRT* sequences, we also constructed four other plasmids based on four other unique *FRT* sequences: pmFRT-PC$_{S12}$-*gat*, with the flanking *FRT* spacer TGTAGATA; pFRT1-PC$_{S12}$-*gat*, with a TCTTGAAA spacer; pFRT2-PC$_{S12}$-*gat*, with a TCTAGGAA spacer; and pFRT3-PC$_{S12}$-*gat*, with a TCTCGAAA spacer. The differences in the spacer sequence yield unique *FRT*s. The unique *FRT*s in these five plasmids (pwFRT-PC$_{S12}$-*gat*, pmFRT-PC$_{S12}$-*gat*, pFRT1-PC$_{S12}$-*gat*, pFRT2-PC$_{S12}$-*gat*, and pFRT3-PC$_{S12}$-*gat*) allow for multiple rounds of allelic replacement by recycling the same marker with Flp-*FRT* excision, reducing the risk of chromosomal deletions and rearrangements as observed previously (1). To construct these four 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 produce plasmid backbones without the Gm^r marker. Each plasmid backbone was digested with EcoRV and XhoI and was ligated with the PC$_{S12}$-*gat* fragment obtained from pwFRT-PC$_{S12}$-*gat* by EcoRV and XhoI digestion. Essentially, the sequences of all four new pFRT-PC$_{S12}$-*gat-FRT* plasmids are the same as that of pwFRT-PC$_{S12}$-*gat*, with the exception of the *FRT* spacer sequences flanking the *gat* cassette. The *FRT*-flanked *bar* cassette on pwFRT-PC$_{S12}$-*bar* was constructed by first amplifying the *bar* gene from pCAMBIA-1301-*bar* via a two-step PCR, as described above for the construction of pBBRMCS1-2-PC$_{S12}$-*bar*. The 800-bp *bar* fragment was digested with XhoI and ligated into EcoRV- and XhoI-digested pwFRT-PC$_{S12}$-Tel^r, replacing the Tel^r-cassette with the *bar* gene to produce pwFRT-PC$_{S12}$-*bar*.

Construction of pBAKA-Δ*asd*$_{Bp}$::*gat-FRT* and pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*. The *B. pseudomallei* K96243 *asd* gene was amplified from chromosomal DNA using oligonucleotides 892 and 893. This *asd*$_{Bp}$ gene sequence is essentially identical for K96243 and 1026b. The 1.4-kb fragment was digested with EcoRI and HindIII and was cloned into pUC18 digested with the same enzymes. After cloning, the purified plasmid, pUC18-*asd*$_{Bp}$, was electroporated into the *dam*-negative strain GM33. Plasmids were isolated, digested with BclI (*dam* methylation sensitive) and EcoRV, and blunt ended. The plasmid backbone was then ligated to the 0.7-kb fragment from SmaI-digested pwFRT-PC$_{S12}$-*gat*, resulting in a 250-bp deletion in the *asd*$_{Bp}$ gene. pUC18-Δ*asd*$_{Bp}$::*gat-FRT* was then digested with EcoRI and HindIII, and the 1.9-kb fragment was cloned into pBAKA, cut with the same enzymes, to produce pBAKA-Δ*asd*$_{Bp}$::*gat-FRT*. The *gat* gene is in the same orientation as the *asd*$_{Bp}$ gene (Fig. 5A).

To construct pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*, the *B. pseudomallei* K96243 *dapB* gene was amplified from chromosomal DNA using oligonucleotides 1048 and 1050. The 1.9-kb fragment was digested with HindIII and XbaI and was ligated

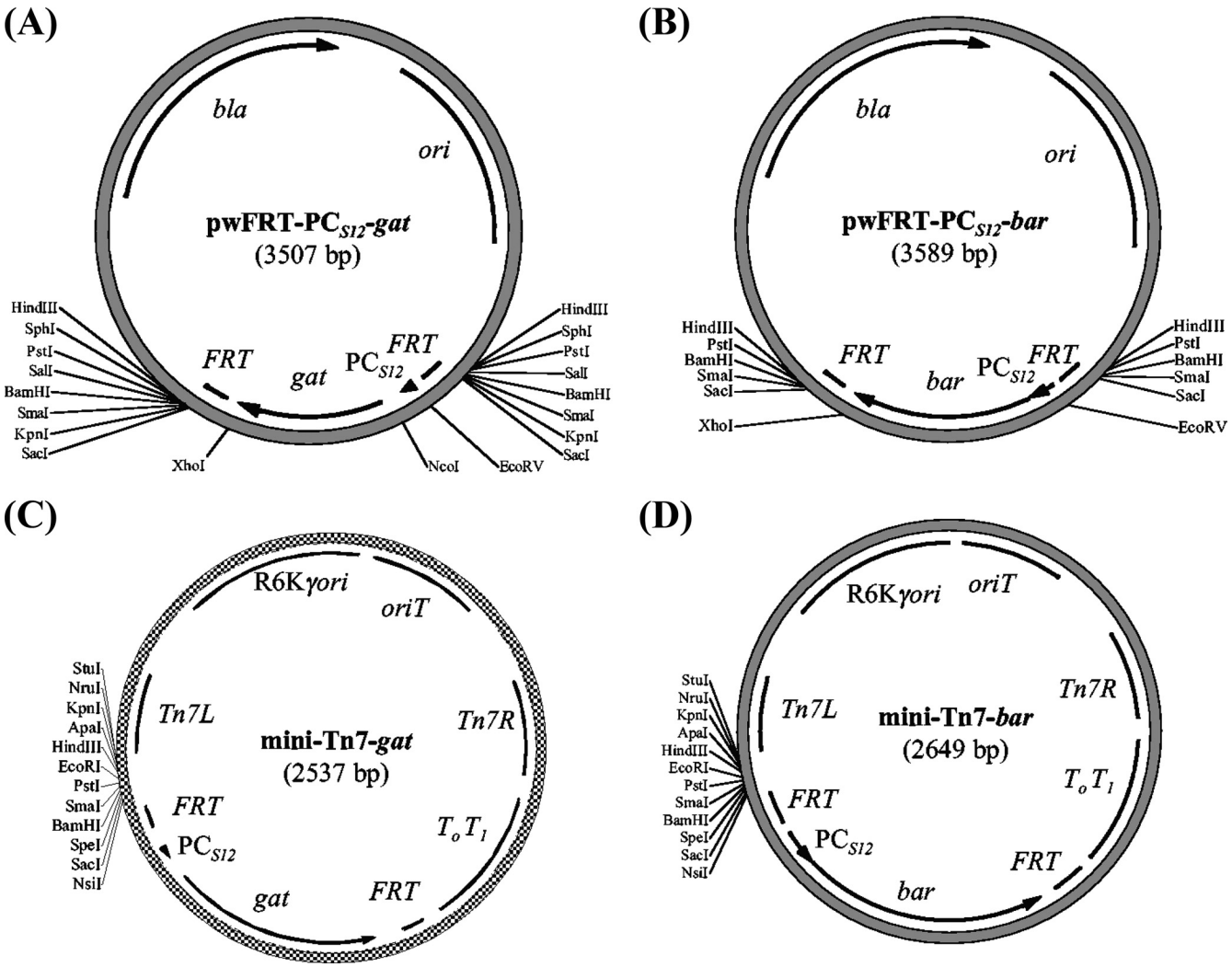

FIG. 4. Maps of pwFRT-PC$_{S12}$-*gat* (A), pwFRT-PC$_{S12}$-*bar* (B), mini-Tn*7*-*gat* (C), and mini-Tn*7*-*bar* (D). (A) pwFRT-PC$_{S12}$-*gat* is flanked with symmetrical restriction-sites (HindIII to SacI) that will cut to remove the *gat* cassette flanked with identical wild-type *FRT* sequences. Not shown are four other *FRT*-*gat* cassettes with unique flanking *FRT*-sequences (pmFRT-*gat*, pFRT1-*gat*, pFRT2-*gat*, and pFRT3-*gat*), where the *gat* marker is flanked by identical *FRT*s with unique spacer sequences. The DNA sequences and restriction sites for all five *gat*-*FRT* cassettes are identical with the exception of the spacers. (B) pwFRT-PC$_{S12}$-*bar*, with *bar* flanked by wild-type *FRT* sequences and symmetrical restriction enzyme sites. (C and D) mini-Tn*7*-*gat* (C) and mini-Tn*7*-*bar* (D) were engineered to allow site-specific integration of the cloned gene(s), using the non-antibiotic resistance *bar* or *gat* selectable marker, with the assistance of a helper plasmid (pTNS3-*asd*$_{Ec}$). *bla*, β-lactamase-encoding gene; *ori*, ColE1 origin of replication; *oriT*, conjugal origin of transfer; R6Kγ*ori*, π protein-dependent R6K origin of replication; *Tn7L* and *Tn7R*, left and right transposase recognition sequences; T_oT_1, transcriptional terminator.

into pBAKA cut with the same enzymes. pBAKA-*dapB*$_{Bp}$ was then digested with SalI and ligated with the 0.7-kb fragment from the SalI digestion of pwFRT-PC$_{S12}$-*gat*, producing pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*. The *gat* gene is in the opposite orientation to the *dapB*$_{Bp}$ gene.

Engineering of *B. pseudomallei* Δ*asd*$_{Bp}$::*gat-FRT* and Δ*dapB*$_{Bp}$::*gat-FRT* mutants. E1354 was utilized as the conjugal donor to introduce the allelic-replacement vectors, pBAKA-Δ*asd*$_{Bp}$::*FRT-gat* and pBAKA-Δ*dapB*$_{Bp}$::*gat-FRT*, into *B. pseudomallei* strain K96243. Conjugations were carried out as described above, and 100 μl and 200 μl of the conjugation mixtures were plated onto MG medium plus 200 μg/ml DAP, 0.3% GS, and 1 mM (each) Lys, Met, and Thr; these last 3 amino acids (3AA) and DAP are required for the specific Δ*asd* mutation (Fig. 5B). Colonies appearing after 3 to 4 days were streaked out on the same medium supplemented with 0.1% cPhe to counterselect against *pheS*. It is critical for clean counterselection that the medium, in the presence of cPhe, contain no competing phenylalanine, as previously described (2). GS-resistant mutants were screened by patching with toothpicks onto plates with and without DAP (MG medium plus 0.3% GS, 0.1% cPhe, and 1 mM 3AA, with or without 200 μg/ml DAP). Mutants unable to grow without DAP were purified once on LB medium plus DAP and were patched again on MG medium plus 0.3% GS, 0.1% cPhe, and 1 mM 3AA, with or without 200 μg/ml DAP, for confirmation. Purification from potential background on LB medium plus DAP is recommended and is very important, because GS is bacteriostatic, rather than bactericidal, at this effective concentration. Further screening and confirmation of DAP-requiring mutants were performed by PCR using oligonucleotides 1062 and 1063, which annealed to the chromosome outside of the region cloned for allelic replacement (Fig. 5A and C).

To engineer the *B. pseudomallei* K96243 Δ*dapB*$_{Bp}$::*gat-FRT* mutant, the methodologies were essentially the same as for the engineering of Δ*asd*$_{Bp}$::*gat-FRT*, except that only 1 mM Lys was added to the medium with DAP rather than the 3AA (Fig. 5B). DAP-requiring colonies were further purified as described above on LB medium plus DAP, because GS is a bacteriostatic agent. Δ*dapB*$_{Bp}$ mutants were screened by PCR using oligonucleotides 1070 and 1071, which anneal outside of the oligonucleotides used for cloning (Fig. 5C).

For *B. pseudomallei* strain 1026b, we engineered and confirmed the Δ*asd*$_{Bp}$

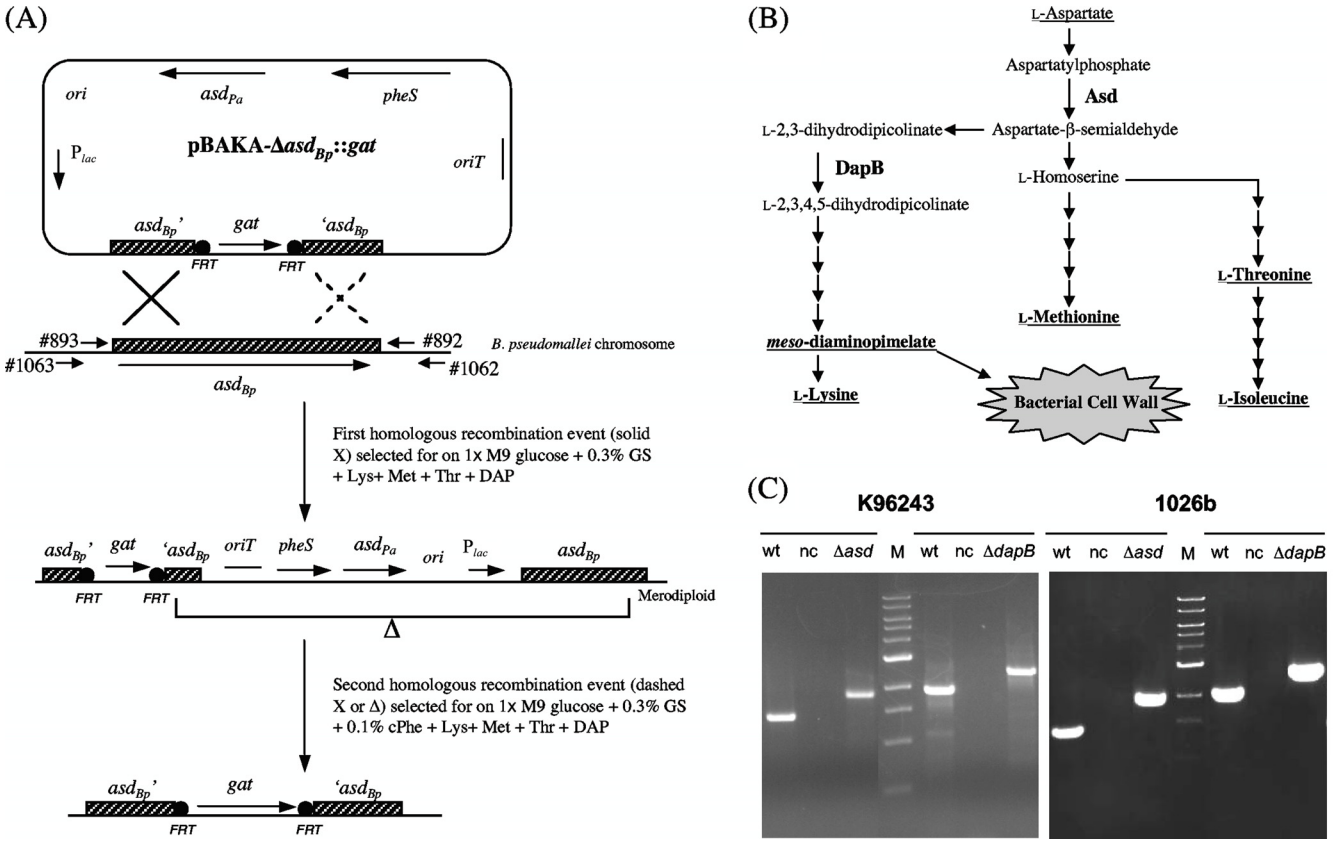

FIG. 5. (A) Gene replacement strategy using a *gat-FRT* cassette to inactivate the *B. pseudomallei* strain K96243 and 1026b asd_{Bp} genes. Oligonucleotides 892 and 893 were used in the initial cloning of the asd_{Bp} gene into the allelic-replacement vector pBAKA, and the asd_{Bp} gene was inactivated with the *gat-FRT* cassette. Deletion of the chromosomal asd_{Bp} gene with pBAKA-Δasd_{Bp}::*gat* was performed as shown. PCR verification of the Δasd_{Bp} mutant was done using outside oligonucleotides 1062 and 1063. The asd_{Bp} genes of both the K96243 and the 1026b strain were inactivated using pBAKA and *pheS* for counterselection. Similarly, the $dapB_{Bp}$ gene of strain K96243 was inactivated using pBAKA and *pheS* for counterselection (not shown) (see Materials and Methods). Oligonucleotides 1049 and 1051 were used to amplify the Δ$dapB_{Bp}$::*gat* cassette from plasmid pBAKA-Δ$dapB_{Bp}$::*gat* in order to inactivate the $dapB_{Bp}$ gene from strain 1026b using the DNA incubation method (46) (see Materials and Methods). (B) Bacterial amino acid biosynthetic pathway of the aspartate family, where aspartate is used to synthesize DAP, Lys, Met, Thr, and Ile. The indicated reactions catalyzed by Asd and DapB are central to this pathway, and mutants of these genes cannot cross-link their cell walls due to the lack of DAP. (C) PCR verification of the Δasd_{Bp} and Δ$dapB_{Bp}$ mutants. In each case, as expected, the PCR products indicated that the chromosomal fragment of the mutant is larger than that of the wild type (wt), and the no-template negative control (nc) showed no PCR product. asd_{Bp}, *B. pseudomallei asd* gene encoding aspartate-semialdehyde dehydrogenase; asd_{Pa}, *P. aeruginosa asd* gene; $dapB_{Bp}$, *B. pseudomallei* gene encoding dihydrodipicolinate reductase; M, 1-kb ladder (New England Biolabs); P*lac*, *lac* promoter; *pheS*, mutant *B. pseudomallei* gene encoding the α subunit of phenylalanyl tRNA synthase.

mutant essentially as described above for strain K96243, via counterselection with cPhe/*pheS*. To demonstrate that the DNA incubation approach also works by using *gat* for selection with GS in strain 1026b, we used the published DNA incubation and natural transformation approach to delete the $dapB_{Bp}$ gene in strain 1026b (46). pBAKA-Δ$dapB_{Bp}$::*gat-FRT* was used as a template along with oligonucleotides 1049 and 1051 in a PCR to obtain a linear 2.7-kb Δ$dapB_{Bp}$::*gat-FRT* fragment. Allelic replacement was performed as previously published (2), but selection was carried out on MG medium plus 0.3% GS, 200 μg/ml DAP, and 1 mM Lys. GS-resistant colonies that required DAP were purified and further confirmed by PCR with oligonucleotides 1070 and 1071 as described above.

Phenotypic lysis of Δ*asd* and Δ*dapB* mutants without DAP. The *B. pseudomallei* wild-type strain K96243, the K96243 Δasd_{Bp}::*gat-FRT* mutant, and the K96243 Δ$dapB_{Bp}$::*gat-FRT* mutant were first grown overnight in LB medium alone (wild-type strain) or LB medium plus DAP (Δasd_{Bp}::*gat-FRT* and Δ$dapB_{Bp}$::*gat-FRT* strains). One milliliter of each culture was centrifuged, and cell pellets were washed twice with LS (LB–no-salt) medium and resuspended in 20 μl of LS. Ten microliters of each concentrated cell resuspension was spotted onto LS plates and LS-plus-DAP plates and was incubated at 37°C. After 18 h, cells were resuspended in sterile saline (0.85% NaCl) and smeared onto glass slides. The slides were then air dried and fixed with 1% paraformalydehyde (in phosphate-buffered saline) for 1 h. This fixing method was initially tested on wild-type K96243, and the slide was incubated in rich LB medium for 3 weeks to ensure that no growth was observed, indicating complete killing. Finally, the cells were stained with safranin for 10 min, gently rinsed with water, and examined under an 100× oil immersion objective lens (Fig. 6).

Construction of mini-Tn*7-bar* and mini-Tn*7-bar-asd*$_{Bp}$. To construct the site-specific mini-Tn*7-bar* transposon, mini-Tn*7*-Tel^r was digested with XbaI (cut in the flanking *FRT* spacer regions), and the *bar* cassette from pwFRT-PC$_{S12}$-*bar* (also digested with XbaI in the *FRT* spacer regions) was ligated to replace the Tel^r cassette. Recovery of the *FRT* sequences was verified by confirming the orientation of the cloned PC$_{S12}$-*bar* fragment, and recovery of the XbaI sites was verified via restriction enzyme digestions. To construct mini-Tn*7-bar-asd*$_{Bp}$, the asd_{Bp} gene with 600 bp of upstream sequence was amplified from the *B. pseudomallei* K96243 chromosome to include the putative promoter. The 1.8-kb asd_{Bp} gene was PCR amplified from K96243 chromosomal DNA using oligonucleotides 893 and 1117, and the product was digested with EcoRI and HindIII. mini-Tn*7-bar* was digested with the same enzymes and ligated to this 1.8-kb asd_{Bp} gene, resulting in mini-Tn*7-bar-asd*$_{Bp}$. The asd_{Bp} gene was cloned in the same orientation as the *bar* cassette. The functionality of the asd_{Bp} gene was verified by transformation into a Δ*asd E. coli* strain (E1345); growth was observed on LB medium in the absence of DAP.

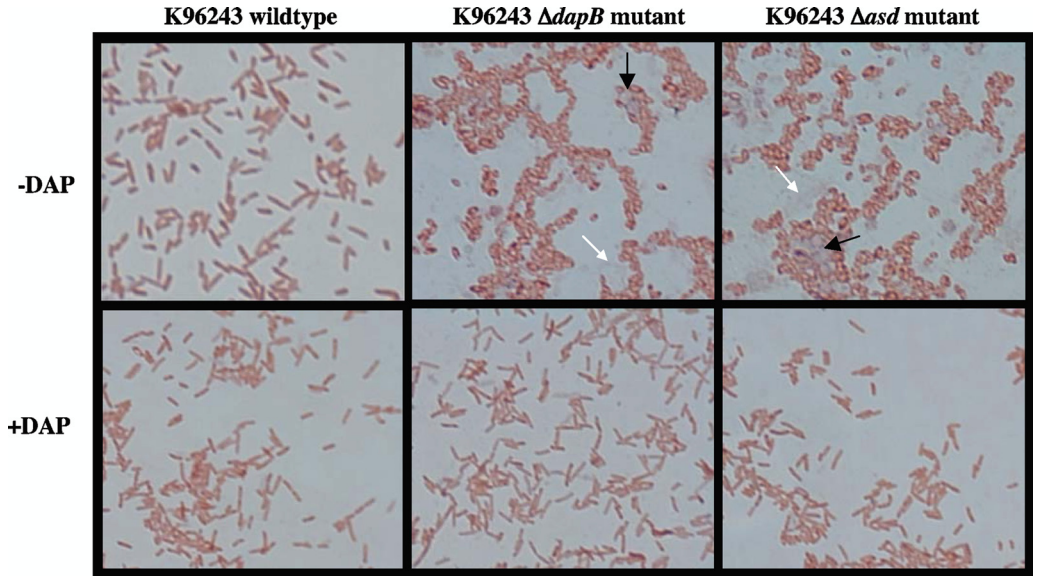

FIG. 6. Phenotypic characterization of *B. pseudomallei* K96243 Δasd_{Bp} and $\Delta dapB_{Bp}$ mutants. Wild-type K96243 was rod shaped when grown in the absence or presence of DAP (left). The Δasd_{Bp} (center) and $\Delta dapB_{Bp}$ (right) mutant strains grow, but "pop and die" without the ability to cross-link their cell walls in the absence of DAP. The majority of the bacteria are in the process of forming protoplasts. Some protoplasts could be observed (black arrows), as well as cell debris (white arrows) due to bacterial lysis; these were absent when mutants were grown in the presence of DAP (bottom).

Complementation of the *B. pseudomallei* Δasd_{Bp}::*gat-FRT* mutant. The *B. pseudomallei* 1026b Δasd_{Bp}::*gat-FRT* mutant was complemented using the mini-Tn*7-bar-*asd_{Bp} vector. The mini-Tn*7-bar-*asd_{Bp} vector and its helper plasmid (pTNS3-asd_{Ec}) were transformed individually into *E. coli* E1354 (a conjugation-proficient tryptophan auxotroph) and conjugated into *B. pseudomallei* Δasd_{Bp}::*gat-FRT* in a triparental mating experiment. Conjugation mixtures were resuspended in 1 ml of 1× M9 minimal medium, and 100 μl of a 1:10 dilution was plated on MG medium plus 200 μg/ml DAP, 2.5% PPT, and 1 mM (each) Lys, Met, and Thr. This medium prevents the *E. coli* donor (Trp auxotroph) from growing and selects for PPT-resistant *B. pseudomallei*. Ten isolates were screened for positive integration using oligonucleotide 876, which anneals in the Tn7L region of the mini-Tn*7-bar* site-specific transposon, and oligonucleotide 1079, 1080, or 1081, each of which is specific for one of the three possible integration sites on the chromosome (10). Two positive isolates with insertions at *glmS1* and three isolates with insertions at *glmS2* were chosen for further characterization (below).

Growth of wild-type *B. pseudomallei* strain 1026b, its Δasd_{Bp}::*gat-FRT* mutant, and Δasd_{Bp}::*gat-FRT*/*attTn7-bar-*asd_{Bp} complemented isolates. To further characterize the Δ*asd* mutation, we first grew strain 1026b, the Δasd_{Bp} mutant strain, and several complemented strains overnight in LB medium plus DAP at 37°C with shaking at 250 rpm. One milliliter of each culture was harvested by centrifugation at 8,000 × *g* for 2 min. The pellet was washed twice with 1× M9 buffer to remove any residual nutrients and was resuspended in 1 ml of 1× M9 buffer. To determine the amino acid auxotrophic properties of these strains, the cell suspensions were diluted 20× in 1× M9 buffer. Five microliters of each culture was spotted onto plates with MG medium plus 200 μg/ml DAP and 1 mM (each) Ile, Lys, Met, and Thr; the same amount of diluted culture was spotted onto five other plates, each missing one of the four amino acids or DAP. Growth on the plates was observed after 24 h and 7 days (Fig. 7).

Nucleotide sequence accession numbers. The sequences of all constructs shown in Fig. 4 were submitted to GenBank. The accession numbers are FJ384986 for pwFRT-PC_{S12}-*gat*, FJ858786 for pwFRT-PC_{S12}-*bar*, FJ858785 for mini-Tn*7-gat*, and FJ826509 for mini-Tn*7-bar*.

RESULTS AND DISCUSSION

Effectiveness of GS against *Burkholderia* species. Although studies have measured the inhibitory concentrations of GS for *P. aeruginosa*, *E. coli*, *B. subtilis*, and *B. japonicum* (16, 55), no studies have determined the GS inhibitory concentrations for *Burkholderia* species. It was previously shown that growth inhibition of *B. japonicum* was observed at a lower GS concentration (5 mM, or 0.085%) and that rapid death occurred at a higher GS concentration (10 mM, or 0.17%) (55). Thus, GS could be bactericidal depending on the concentration used. We initially determined the inhibitory or killing action of GS for three *Burkholderia* select-agent strains (Fig. 2). When exposed to different concentrations of GS for 24 h, *B. mallei* was found to be more sensitive to GS than *B. pseudomallei*, and >60% death was observed upon exposure to a concentration as low as 0.25%. Clearly, compared to the high cell density following overnight incubation in the absence of GS, no significant replication beyond doubling was observed at concentrations as low as 0.25% GS. Both *B. pseudomallei* strains replicated to slightly less than double their original number at 0.25% GS and were killed at higher concentrations of GS (Fig. 2), probably because of residual intracellular aromatic amino acid levels after growth in LB medium prior to GS exposure. Although the mechanism of GS inhibition of *Burkholderia* species is to be determined in future studies, it is likely similar to the mechanism of EPSPS inhibition in plants, which has been confirmed for *P. aeruginosa*, *E. coli*, *B. subtilis*, and *B. japonicum* (16, 55).

We next wanted to determine the MICs of GS for members of the *Burkholderia* genus before empirically identifying the GS_{EC}s in liquid and on solid media (see Materials and Methods). Significantly high cell densities, typical in genetic manipulations (e.g., 10^5 CFU was added to liquid medium and 100 μl of ~10^9 CFU/ml was plated onto solid medium containing different concentrations of GS), were inoculated to determine the MIC of GS as the concentration at which no growth was observed after 2 days (liquid medium) or at which no spontaneously resistant colonies arose after 2 weeks (solid medium). The GS_{EC} above the GS MIC was defined and utilized to

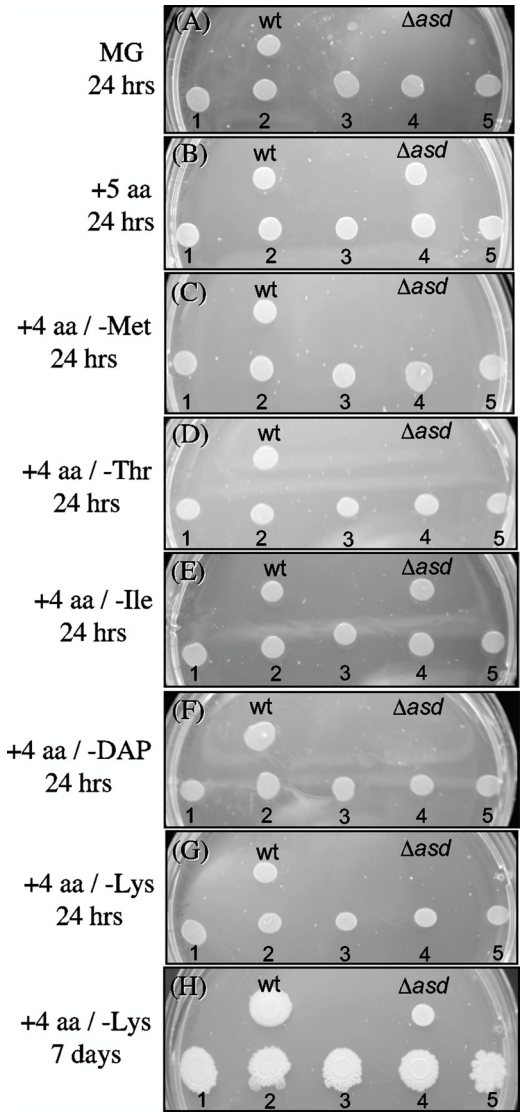

FIG. 7. Growth characteristics of the *B. pseudomallei* K96243 Δasd_{Bp} mutant and five complemented isolates relative to that of the wild type (wt) on medium lacking amino acids (aa) of the aspartate family. (A) On 1× MG medium, the Δasd_{Bp} mutant did not grow compared to the wt, whereas five strains (numbered 1 to 5) complemented using the mini-Tn*7*-*bar*-asd_{Bp} transposon all grew as well as the wt. Spots 1 and 2 are Tn*7*-*bar*-asd_{Bp}-complemented isolates transposed at the *glmS1* site, while spots 3 to 5 are complemented isolates transposed at the *glmS2* site. (B) The Δasd_{Bp} mutant grew similarly to the wt on MG medium when provided with all five aa of the aspartate family (DAP, Lys, Met, Thr, and Ile). (C through F) The Δasd_{Bp} mutant could not grow when four of the five aa were present in the MG medium and only Met (C), Thr (D), or DAP (F) was omitted, whereas the wt and all complemented strains grew well on these media. The Δasd_{Bp} mutant still grew when Ile was omitted from MG medium containing the other four aa (E), because Thr in the medium could be converted to Ile in this pathway. (G) Surprisingly, no growth was observed when Lys was omitted from the MG medium supplemented with four aa (Met, Thr, DAP, and Ile). We suspect that the amount of DAP provided was shuffled for use in cell wall biosynthesis and that very little was converted to Lys for growth. The Δasd_{Bp} mutant grew slowly on this medium. (H) When the plate in panel G was incubated for another 6 days, growth was observed for the Δasd_{Bp} mutant, indicating that some DAP did get converted to Lys. All other plates on which the Δasd_{Bp} mutant did not grow after 1 day also did not show growth of this mutant after 7 days (data not shown).

ensure no growth of high inocula in liquid medium after 4 days or no growth for 3 weeks on solid medium (Table 4). We determined the GS_{EC} within this time frame for liquid and solid media, because this period is sufficient to observe most mutants that will arise during allelic replacement and also allows most *Burkholderia* species to grow on minimal medium during selection. The GS_{EC}s for *E. coli* and for *B. pseudomallei*, *B. mallei*, and *B. cenocepacia* K56-2 are higher than those for other *Burkholderia* species (Table 4). We have utilized the GS_{EC} in Table 4 to select for the *gat* gene (see below) successfully in *E. coli*, *B. thailandensis*, and the two wild-type *B. pseudomallei* strains. Thus, we are confident that the GS_{EC}s for other species in Table 4 are appropriate.

Roundup is an appropriate source of GS for selection. We have not encountered any problem with the solubility of GS at high concentrations (10% was the highest concentration tested using purified GS). Indeed, the "superconcentrated" Roundup that we purchased contained 50% GS in aqueous solution. In addition to this advantage, GS is readily available, inexpensive, relatively nontoxic, and not in clinical use, and it gives tight selection (see below). Although purified GS could be purchased from Sigma and other distributors, we do not have concerns with using Roundup for selection, since purified GS gave the same GS_{EC} (data not shown). One bottle of "superconcentrated" Roundup purchased from a local garden supply store has lasted for the duration of this study. Roundup formulations with lower concentrations of GS are also available, although we recommend the "superconcentrated" Roundup, because it is potent enough for >150 liters of culture when used at a final concentration of 0.3%. Selection of pBBR1MCS-2-PC_{S12}-*gat* (kanamycin and GS resistance) in *E. coli* and *B. thailandensis* on kanamycin or on GS from Roundup yielded the same number of colonies (data not shown), providing evidence that Roundup is appropriate for selection. This indicates that no other ingredient(s) in Roundup has adverse effects on the selection of *gat*-containing constructs and that this source of GS is appropriate for selective media.

Engineering of a *gat* cassette and effective selection with GS. We engineered the *gat* gene through GenScript Corporation, based on the previously described GAT protein sequences (5), using an approach similar to that for the synthesis of the *pheS* gene (2). We utilized this approach to optimize the codon usage for efficient expression in *Burkholderia* species, while eliminating many restriction sites within the gene and strategically placing others at certain locations for future manipulation. The engineered *gat* cassette, including the *B. cenocepacia* promoter of the *rpsL* gene (PC_{S12}), is only 563 bp (Fig. 3). The small size of the *gat* cassette makes it easy to manipulate, clone, and amplify by PCR for use in the DNA incubation method of allelic replacement in naturally competent *Burkholderia* species (46). As proof of concept, we utilized this cassette in the DNA incubation approach to delete the *asd* gene of *B. thailandensis* (asd_{Bt}) at its GS_{EC} (data not shown). This confirmed that the GS_{EC}s in Table 4 are sufficient for selection.

Deletional mutagenesis of the essential asd_{Bp} and $dapB_{Bp}$ genes using GS and *gat*. The reliability of any marker for mutagenesis would best be demonstrated by the successful mutagenesis of essential genes. Therefore, we chose two essential genes, *asd* and *dapB*, that are absolutely required for

DAP synthesis and cell wall cross-linking in most gram-negative bacteria (Fig. 5B) (8, 11, 20, 22, 37). Mutation of the *asd* gene makes gram-negative bacteria auxotrophic for three amino acids (Thr, Met, DAP), while *dapB* mutants require only DAP. Although Lys and Ile are also made from the same pathway, the DAP and Thr provided should act as precursors for Lys and Ile biosynthesis, respectively (Fig. 5B). The asd_{Bp} and $dapB_{Bp}$ genes encode aspartate-semialdehyde dehydrogenase (BPSS1704 on chromosome 2) and a dihydrodipicolinate reductase (BPSL2941 on chromosome 1), respectively.

To knock out the asd_{Bp} and $dapB_{Bp}$ genes, we engineered *gat-FRT* cassettes for allelic replacement (Fig. 4A). As mentioned above, a *gat-FRT* cassette was successfully utilized to delete the asd_{Bt} gene using the DNA incubation and natural transformation method (46), where selection with 0.04% GS yielded Δasd_{Bt} mutation frequencies of ~80% (data not shown). We then utilized pBAKA and the *pheS* counterselection approach as previously described (2), with the *gat-FRT* cassette from pwFRT-PC_{S12}-*gat*, to inactivate the asd_{Bp} and $dapB_{Bp}$ genes in *B. pseudomallei* (Fig. 4A and 5A). Independent merodiploids resulting from the first recombination in strain K96243 were obtained with 0.3% GS after 3 days of growth (see Materials and Methods). Streaking of merodiploids onto medium containing 0.1% cPhe and 0.3% GS for counterselection to resolve the mutations yielded DAP-requiring colonies at frequencies of ~25% and ~80% for the *B. pseudomallei* K96243 Δ*asd* and Δ*dapB* mutants, respectively. To demonstrate this principle of allelic replacement with another *B. pseudomallei* strain, we utilized the same approach with cPhe/*pheS* counterselection to create a 1026b Δasd_{Bp} mutant, which yielded a lower mutation frequency of ~10% for this essential gene. Since strain 1026b is also naturally competent, we wanted to utilize the published DNA incubation method for allelic replacement (46) by engineering a Δ$dapB_{Bp}$ mutant, yielding 1026b Δ$dapB_{Bp}$ mutants at a frequency of ~25%. We confirmed these mutations by PCR with oligonucleotides annealing to chromosomal regions outside of the initial primers used for cloning (Fig. 5A and C). Because GS is bacteriostatic at the concentration used (Table 4 and Fig. 2), it is critical to purify all mutants from the potential background contamination before reconfirmation of the phenotype, PCR confirmation, and growth for long-term storage at −80°C. Phenotypically, Δ$dapB_{Bp}$ mutants required DAP for growth (data not shown), while Δasd_{Bp} mutants required DAP, Thr, and Met (Fig. 7). Using wild-type strain K96243 and its mutants as examples, we further characterized the phenotypes of Δasd_{Bp} and Δ$dapB_{Bp}$ mutants. In the presence of DAP, both Δasd_{Bp} and Δ$dapB_{Bp}$ mutants displayed a normal rod-shaped cellular morphology (Fig. 6). However, in the absence of DAP, these two mutants, lacking DAP for cell wall biosynthesis and cross-linking, showed "cell-rounding" characteristics and evidence of lysis (Fig. 6).

Δasd_{Bp} mutant complementation with a site-specific mini-Tn*7-bar-*asd_{Bp} transposon. We engineered a site-specific transposon based on mini-Tn*7*, which has previously been demonstrated to integrate at three possible *glmS* sites in the *B. pseudomallei* chromosome (10) (Fig. 4D and 8A). Our construct, mini-Tn*7-bar*, is based on the nonantibiotic *bar* gene, which encodes resistance to bialaphos and PPT (a bialaphos degradation product also known as glufosinate). Since bialaphos can be very expensive, a cheaper alternative, PPT, can be used. We determined the PPT_{EC}s for *B. mallei* and *B. pseudomallei* to be ~2.5%. Many herbicide brands (e.g., Basta, Buster, Dash, Finale, Hayabusa, Ignite, Conquest, Liberty, Rely, Shield, Harvest, Sweep, and Arise) contain PPT as the active ingredient. Since the PPT_{EC} is quite high, we picked the herbicide Finale, because it contains the highest PPT concentration (11.33%, wt/vol) we could find, although other brands not available on our island (e.g., Liberty and Ignite) can contain 20 to 25% PPT. In this study, we utilized the 11.33% PPT in Finale as the working stock to make media at the 2.5% PPT_{EC}. As proof of concept, we introduced the mini-Tn*7-bar-*asd_{Bp} construct into the *B. pseudomallei* 1026b Δasd_{Bp} strain to complement the Δasd_{Bp} mutation. The suicidal helper plasmid pTNS3-asd_{Ec}, harboring the *E. coli asd* (asd_{Ec}) gene for maintenance in an *E. coli* Δ*asd* strain, aids the transposition of the Tn*7-bar-*asd_{Bp} transposon to one of three possible *glmS* chromosomal targets (Fig. 8A). We selected PPT-resistant colonies in the presence of DAP, Lys, Met, and Thr to prevent bias in immediately selecting for complemented strains. After colonies were patched onto DAP-supplemented medium, it was found that ~64% of PPT-resistant colonies tested (45 out of 70) had been complemented and did not require DAP, while the remaining ~36% were probably spontaneously PPT resistant colonies. It was further confirmed that the majority of the complemented isolates (8 out of 10 tested) had mini-Tn*7-bar-*asd_{Bp} transposed to the region downstream of the *glmS2* target, while 2 out of 10 recombined at the *glmS1* target (Fig. 8B). No transposition at the *glmS3* target was observed. These data indicated that the PPT in Finale was appropriate for selection of the *bar* gene and yielded a fairly high frequency of transposition.

To further characterize five complemented isolates along with the wild-type strain 1026b and the Δasd_{Bp} mutant, we spotted these strains onto various media lacking one of the five amino acids (DAP, Lys, Met, Thr, or Ile) in the aspartate family of amino acid biosynthetic pathways. Media lacking Met, Thr, or DAP yielded no growth of the Δasd_{Bp} mutant compared to the growth of the wild-type strain 1026b and the five complemented strains, confirming that the Asd reaction gives rise to these amino acids (Fig. 5B and 7). Ile and Lys were not required by the Δasd_{Bp} mutant, since Thr and DAP will yield Ile and Lys, respectively (Fig. 7E and H). In summary, the Δ*asd* mutant of *B. pseudomallei* displayed a phenotype similar to those of *asd* mutants of other gram-negative bacteria, and the successful complementation of this mutant suggests that our allelic-replacement approach did not introduce any undesirable mutations by selection with Roundup and Finale.

Conclusions. (i) We engineered and successfully demonstrated the use of a novel non-antibiotic resistance *gat* marker, based on resistance to GS, in *Burkholderia* species. This cassette was demonstrated to be useful for allelic replacement of essential genes in *B. pseudomallei*, adding valuably to the limited number of select-agent approved markers. The advantages of using GS-containing herbicides to select for the *gat* cassette in recombinant work include cost-effectiveness, availability, low toxicity, no clinical use, high solubility, relatively tight selection, and the small size of the *gat* marker. The *gat* cassette was used successfully in more than one allelic-replacement strategy to delete two essential genes, confirming its value, the

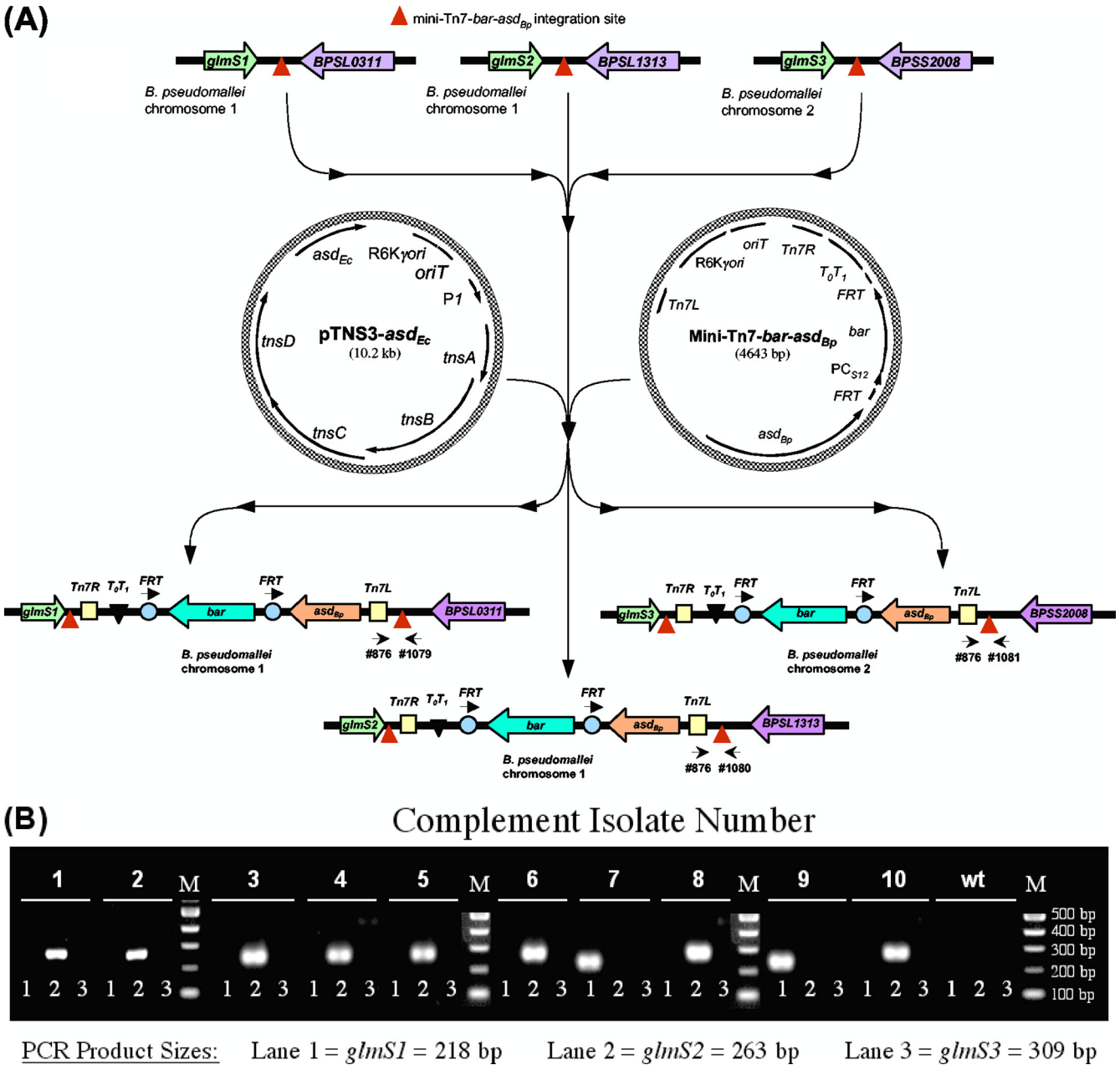

FIG. 8. Single-copy complementation of the *B. pseudomallei* 1026b Δ*asd*$_{Bp}$ mutant using mini-Tn*7-bar-asd*$_{Bp}$. (A) The suicidal plasmid mini-Tn*7-bar-asd*$_{Bp}$ and its suicidal helper plasmid, pTNS3-*asd*$_{Ec}$, were introduced into the *B. pseudomallei* 1026b Δ*asd*$_{Bp}$ mutant by conjugation. Tn*7* has three possible integration sites on different chromosomes (indicated by red triangles), as previously described (10), which can result in complementation of the Δ*asd*$_{Bp}$ mutation from three different chromosomal loci, as depicted according to the annotation of *B. pseudomallei* strain K96243. Ten random complemented isolates were screened using oligonucleotide Tn7L (876) and an oligonucleotide specific for each potential integration site (oligonucleotide 1079, 1080, or 1081), as indicated by arrows. (B) For each isolate, PCR verification of 10 random complemented isolates was performed for all three *glmS* sites (lanes 1, 2, and 3). Insertion downstream of *glmS1* would result in a 218-bp PCR product; insertion downstream of *glmS2* would result in a 263-bp fragment; and insertion downstream of *glmS3* would result in a 309-bp PCR product. Isolates 1, 2, 3, 4, 5, 6, 8, and 10 had Tn*7* inserted downstream of *glmS2.* Isolates 7 and 9 showed PCR products near 200 bp, indicating Tn*7* integration downstream of *glmS1*. P1, P1 integron promoter; *glmS1*, *glmS2*, and *glmS3* encode three different *B. pseudomallei* glucosamine 6-phosphate synthetases; M, 100-bp ladder (New England Biolabs); *tnsABCD*, Tn*7* transposase-encoding genes.

usefulness of *pheS* as a counterselectable marker, and compatibility with the DNA incubation method for naturally competent *Burkholderia* species (46). (ii) We initiated the successful utilization of a second non-antibiotic resistance marker, based on the better-characterized *bar* gene (49), encoding bialaphos and PPT (glufosinate) resistance. This will hopefully also expand the future use of this marker for select-agent species. One minor disadvantage of using *gat* and *bar* is the requirement for minimal media lacking two of the three aromatic amino acids (Phe, Tyr, or Trp) and glutamine, respectively.

Therefore, for most mutations, the use of 1× M9 medium plus 20 mM glucose should suffice for *B. pseudomallei*, *B. mallei*, and *B. thailandensis*. Note that we added DAP, Lys, Met, and Thr to the media in this study, because of mutant-specific amino acid requirements (e.g., Δasd_{Bp} and $\Delta dapB_{Bp}$). (iii) We created two mutants in two wild-type *B. pseudomallei* strains, which may be promising as future attenuated vaccine candidates, since DAP is a bacterium-specific product not available in mammalian hosts. (iv) Finally, it should be noted that all genetic tools used in this study are completely devoid of antibiotic resistance during introduction and selection. The potential use of *gat* and *bar* may be expanded to other select-agent species (e.g., *Brucella* and *Francisella* spp.), since minimal media lacking Phe, Tyr, Trp, and Gln have been defined for some of these species (6, 18). GS and PPT, compounds originally designed to kill plant weeds, may prove quite useful for the future selection of recombinants in bacterial select-agent species.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant R21-AI074608 to T.T.H. A graduate stipend for M.H.N. was supported by an NSF IGERT award (0549514) to B.A.W.

REFERENCES

1. **Barekzi, N., K. L. Beinlich, T. T. Hoang, X.-Q. Pham, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2000. The *oriC*-containing region of the *Pseudomonas aeruginosa* chromosome undergoes large inversions at high frequency. J. Bacteriol. **182:**7070–7074.
2. **Barrett, A. R., Y. Kang, K. S. Inamasu, M. S. Son, J. M. Vukovich, and T. T. Hoang.** 2008. Genetic tools for allelic replacement in *Burkholderia* species. Appl. Environ. Microbiol. **74:**4498–4508.
3. **Borgherini, G., P. Poubeau, F. Paganin, S. Picot, A. Michault, F. Thibault, and C. A. Berod.** 2006. Melioidosis: an imported case from Madagascar. J. Travel Med. **13:**318–320.
4. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48:**317–320.
5. **Castle, L. A., D. L. Siehl, R. Gorton, P. A. Patten, Y. H. Chen, S. Bertain, H.-J. Cho, N. Duck, J. Wong, D. Liu, and M. W. Lassner.** 2004. Discovery and directed evolution of a glyphosate tolerance gene. Science **304:**1151–1154.
6. **Chamberlain, R. E.** 1965. Evaluation of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. **13:**232–235.
7. **Chan, Y. Y., and K. L. Chua.** 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. J. Bacteriol. **187:**4707–4719.
8. **Chen, N. Y., S. Q. Jiang, D. A. Klein, and H. Paulus.** 1993. Organization and nucleotide sequence of the *Bacillus subtilis* diaminopimelate operon, a cluster of genes encoding the first three enzymes of diaminopimelate synthesis and dipicinolate synthase. J. Biol. Chem. **268:**9448–9465.
9. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. **18:**383–416.
10. **Choi, K. H., T. Mima, Y. Casart, D. Rholl, A. Kumar, I. R. Beacham, and H. P. Schweizer.** 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. Appl. Environ. Microbiol. **74:**1064–1075.
11. **Cirillo, J. D., T. R. Weisbrod, L. Pascopella, B. R. Bloom, and W. R. Jacob, Jr.** 1994. Isolation and characterization of the aspartokinase and aspartate semialdehyde dehydrogenase operon from mycobacteria. Mol. Microbiol. **11:**629–639.
12. **Dance, D. A. B.** 1991. Melioidosis: the tip of the iceberg? Clin. Microbiol. Rev. **4:**52–60.
13. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.
14. **Dill, G. M.** 2005. Glyphosate-resistance crops: history, status, and future. Pest Manag. Sci. **61:**219–224.
15. **Dorman, S. E., V. J. Gill, J. I. Gallin, and S. M. Holland.** 1998. *Burkholderia pseudomallei* infection in a Puerto Rican patient with chronic granulomatous disease: case report and review of occurrences in the Americas. Clin. Infect. Dis. **26:**889–894.
16. **Fischer, R., A. Berry, C. G. Gaines, and R. A. Jensen.** 1986. Comparative action of glyphosate as a trigger of energy drain in eubacteria. J. Bacteriol. **168:**1147–1154.
17. **Frischknecht, F.** 2003. The history of biological warfare. Eur. Mol. Biol. Org. **4:**S47–S52.
18. **Gerhardt, P., and J. B. Wilson.** 1948. The nutrition of brucellae: growth in simple chemically defined media. J. Bacteriol. **56:**17–24.
19. **Hamad, M. A., S. L. Zajdowicz, R. K. Holmes, and M. I. Voskuil.** 2009. An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*. Gene **430:**123–131.
20. **Hatten, L.-A., H. P. Schweizer, N. Averill, L. Wang, and A. B. Schryvers.** 1993. Cloning and characterization of the *Neisseria meningitidis asd* gene. Gene **129:**123–128.
21. **Herrero, M., V. De Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
22. **Hoang, T., S. Williams, H. P. Schweizer, and J. S. Lam.** 1997. Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa asd* gene encoding aspartate-β-semialdehyde dehydrogenase. Microbiology **143:**899–907.
23. **Holden, M. T. G., R. W. Titball, S. J. Peacock, A. M. Cerdeño-Tárraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. F. Oyston, and J. Parkhill.** 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. USA **101:**14240–14245.
24. **How, H. S., K. H. Ng, H. P. Tee, and A. Shah.** 2005. Pediatric melioidosis in Pahang, Malaysia. J. Microbiol. Immunol. Infect. **38:**314–319.
25. **Issack, M. I., C. D. Bundhun, and H. Gokhool.** 2005. Melioidosis in Mauritius. Emerg. Infect. Dis. **11:**139–140.
26. **Jacob, G. S., J. R. Garbow, L. E. Hallas, N. M. Kimack, G. M. Kinshore, and J. Schaefer.** 1988. Metabolism of glyphosate in *Pseudomonas* sp. strain LBr. Appl. Environ. Microbiol. **54:**2953–2958.
27. **Jäderlund, L., M. Hellman, I. Sundh, M. J. Bailey, and J. K. Jansson.** 2008. Use of a novel nonantibiotic triple marker gene cassette to monitor high survival of *Pseudomonas fluorescens* SBW25 on winter wheat in the field. FEMS Microbiol. Ecol. **63:**156–168.
28. **Jesudason, M. V., A. Anbarasu, and T. J. John.** 2003. Septicaemic melioidosis in a tertiary care hospital in south India. Indian J. Med. Res. **117:**119–121.
29. **Kang, Y., M. H. Norris, A. R. Barrett, B. A. Wilcox, and T. T. Hoang.** 2009. Engineering of tellurite-resistant genetic tools for single-copy chromosomal analysis of *Burkholderia* spp. and characterization of the *Burkholderia thailandensis betBA* operon. Appl. Environ. Microbiol. **75:**4015–4027.
30. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
31. **Liu, C.-M., P. A. McLean, C. C. Sookdeo, and F. C. Cannon.** 1991. Degradation of the herbicide glyphosate by members of the family *Rhizobiaceae*. Appl. Environ. Microbiol. **57:**1799–1804.
32. **Marinus, M. G., and N. R. Morris.** 1974. Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K-12. J. Mol. Biol. **85:**309–322.
33. **Moore, R. A., D. DeShazer, S. Reckseidler, A. Weissman, and D. E. Woods.** 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. **43:**465–470.
34. **Nierman, W. C., D. DeShazer, H. S. Kim, H. Tettelin, K. E. Nelson, T. Feldblyum, R. L. Ulrich, C. M. Ronning, L. M. Brinkac, S. C. Daugherty, T. D. Davidsen, R. T. Deboy, G. Dimitrov, R. J. Dodson, A. S. Durkin, M. L. Gwinn, D. H. Haft, H. Khouri, J. F. Kolonay, R. Madupu, Y. Mohammoud, W. C. Nelson, D. Radune, C. M. Romero, S. Sarria, J. Selengut, C. Shamblin, S. A. Sullivan, O. White, Y. Yu, N. Zafar, L. Zhou, and C. M. Fraser.** 2004. Structural flexibility in the *Burkholderia mallei* genome. Proc. Natl. Acad. Sci. USA **101:**14246–14251.
35. **Orellana, C.** 2004. Melioidosis strikes Singapore. Lancet Infect. Dis. **4:**655.
36. **Padgette, S. R., D. B. Re, G. F. Barry, D. E. Eichholtz, X. Delannay, R. L. Fuchs, G. M. Kinshore, and R. T. Fraley.** 1996. New weed control opportunities: development of soybeans with a Roundup Ready gene, p. 53–84. *In* S. O. Duke (ed.), Herbicide-resistant crops. CRC Press, Boca Raton, FL.
37. **Pavelka, M. S., Jr.** 2007. Another brick in the wall. Trends Microbiol. **15:**147–149.
38. **Peñaloza-Vazquez, A., G. L. Mena, L. Herrera-Estrella, and A. M. Bailey.** 1995. Cloning and sequencing of the genes involved in glyphosate utilization by *Pseudomonas pseudomallei*. Appl. Environ. Microbiol. **61:**538–543.
39. **Phetsouvanh, R., S. Phongmany, P. Newton, M. Mayxay, A. Ramsay, V.**

Wuthiekanun, and N. J. White. 2001. Melioidosis and Pandora's box in the Lao People's Democratic Republic. Clin. Infect. Dis. **32:**653–654.
40. **Poole, K.** 2008. Bacterial multidrug efflux-pumps serve other functions. Microbe **3:**179–185.
41. **Rholl, D. A., L. A. Trunck, and H. P. Schweizer.** 2008. In vivo Himar1 transposon mutagenesis of *Burkholderia pseudomallei*. Appl. Environ. Microbiol. **74:**7529–7535.
42. **Rolim, D. B.** 2005. Melioidosis, northeastern Brazil. Emerg. Infect. Dis. **11:**1458–1460.
43. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.
44. **Sanchez-Romero, J. M., R. Diaz-Orejas, and V. De Lorenzo.** 1998. Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. Appl. Environ. Microbiol. **64:**4040–4046.
45. **Schweizer, H. P., and S. J. Peacock.** 2008. Antimicrobial drug-selection markers for *Burkholderia pseudomallei* and *B. mallei*. Emerg. Infect. Dis. **14:**1689–1692.
46. **Thongdee, M., L. A. Gallagher, M. Schell, T. Dharakul, S. Songsivilai, and C. Manoil.** 2008. Targeted mutagenesis of *Burkholderia pseudomallei* and *Burkholderia thailandensis* through natural transformation of PCR fragments. Appl. Environ. Microbiol. **74:**2985–2989.
47. **Vandamme, P., J. Govan, and J. LiPuma.** 2007. Diversity and role of *Burkholderia* spp., p. 1–28. *In* T. Coenye and P. Vandamme (ed.), *Burkholderia*: molecular microbiology and genomics. Horizon Scientific, Wymondham, United Kingdom.
48. **Vasil, I. K.** 1996. Phosphinithricin-resistant crops, p. 85–91. *In* S. O. Duke (ed.), Herbicide-resistant crops. CRC Press, Boca Raton, FL.
49. **Wehrmann, A., A. V. Vliet, C. Opsomer, J. Botterman, and A. Schulz.** 1996. The similarities of *bar* and *pat* gene products make them equally applicable for plant engineering. Nat. Biotechnol. **14:**1274–1278.
50. **Whitlock, G. C., D. M. Estes, and A. G. Torres.** 2007. Glanders: off to the races with *Burkholderia mallei*. FEMS Microbiol. Lett. **277:**115–122.
51. **Wiersinga, W. J., T. van der Poll, N. J. White, N. P. Day, and S. J. Peacock.** 2006. Melioidosis: insight into the pathogenicity of *Burkholderia pseudomallei*. Nat. Rev. Microbiol. **4:**272–282.
51a. **Wilson, D. E., and L. C. Chosewood.** 2007. Biosafety in microbiological and biomedical laboratories, 5th ed. Centers for Disease Control and Prevention, Atlanta, GA.
52. **Wuthiekanun, V., N. Pheaktra, H. Putchhat, L. Sin, B. Sen, V. Kumar, S. Langla, S. J. Peacock, and N. P. Day.** 2008. *Burkholderia pseudomallei* antibodies in children, Cambodia. Emerg. Infect. Dis. **14:**301–303.
53. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
54. **Yu, M., and J. S. H. Tsang.** 2006. Use of ribosomal promoters from *Burkholderia cenocepacia* and *Burkholderia cepacia* for improved expression of transporter protein in *Escherichia coli*. Protein Expr. Purif. **49:**219–227.
55. **Zablotowicz, R. M., and K. N. Reddy.** 2004. Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: a minireview. J. Environ. Qual. **33:**825–831.