Published in final edited form as:

Gene. 2009 February 1; 430(1-2): 123–131. doi:10.1016/j.gene.2008.10.011.

An Allelic Exchange System for Compliant Genetic Manipulation of the Select Agents *Burkholderia pseudomallei* and *Burkholderia mallei*

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Abstract

Burkholderia pseudomallei and *B. mallei* are Gram-negative bacterial pathogens that cause melioidosis in humans and glanders in horses, respectively. Both bacteria are classified as category B select agents in the United States. Due to strict select-agent regulations, the number of antibiotic selection markers approved for use in these bacteria is greatly limited. Approved markers for *B. pseudomallei* include genes encoding resistance to kanamycin (Km), gentamicin (Gm), and zeocin (Zeo); however, wild type *B. pseudomallei* is intrinsically resistant to these antibiotics. Selection markers for *B. mallei* are limited to Km and Zeo resistance genes. Additionally, there are few well developed counter-selection markers for use in *Burkholderia*. The use of SacB as a counter-selection method has been of limited success due to the presence of endogenous *sacBC* genes in the genomes of *B. pseudomallei* and *B. mallei*. These impediments have greatly hampered the genetic manipulation of *B. pseudomallei* and *B. mallei* and currently few reliable tools for the genetic manipulation of *Burkholderia* exist. To expand the repertoire of genetic tools for use in *Burkholderia*, we developed the suicide plasmid pMo130, which allows for the compliant genetic manipulation of the select agents *B. pseudomallei* and *B. mallei* using allelic exchange. pMo130 harbors an *aphA* gene which allows for Km selection, the reporter gene *xylE*, which allows for reliable visual detection of *Burkholderia* transformants, and carries a modified *sacB* gene that allows for the resolution of co-integrants. We employed this system to generate multiple unmarked and in-frame mutants in *B. pseudomallei*, and one mutant in *B. mallei*. This vector significantly expands the number of available tools that are select-agent compliant for the genetic manipulation of *B. pseudomallei* and *B. mallei*.

Keywords

Burkholderia pseudomallei; *B. mallei*; allelic exchange

1. Introduction

The Gram-negative bacteria *Burkholderia mallei* and *Burkholderia pseudomallei* are the etiological agents of glanders and melioidosis, respectively (Gilad, 2007). *B. mallei* is a host adapted pathogen that primarily infects solipeds including horses, donkeys, and mules (Neubauer et al., 2005; Whitlock et al., 2007). Human infections with *B. mallei* are rare and

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are associated with close contact with infected animals (Lehavi et al., 2002). *B. pseudomallei* is a saprophytic free-living bacterium that is endemic to wet soil and stagnant water of Southeast Asia and Northern Australia (Dance, 2000; Cheng and Currie, 2005). Human infections with *B. pseudomallei* are commonly restricted to endemic areas and are associated with environmental exposure through inhalation, ingestion, or direct inoculation (Cheng and Currie, 2005). Treatment of human melioidosis remains challenging due to the high level of resistance of *B. pseudomallei* to a wide range of antibiotics (Chaowagul, 2000). Standard antibiotic therapy of human melioidosis requires long treatment regimens and relapse is common (Cheng and Currie, 2005; Limmathurotsakul et al., 2006). Furthermore, melioidosis is increasingly recognized as an emergent infectious disease, in part due to the global travel of both humans and animals (Dance, 2000; Rolim et al., 2005; Inglis et al., 2006; Jones et al., 2008). Both *B. mallei* and *B. pseudomallei* can cause fatal infections in humans and currently there is no approved protective vaccine against these pathogens (Cheng and Currie, 2005; Wiersinga et al., 2006). These issues have raised concerns regarding the use of *B. pseudomallei* and *B. mallei* in biological warfare and have led to the classification of these pathogens as category B select agents in the United States (Rotz et al., 2002; Cheng et al., 2005).

Select-agent guidelines greatly restrict the introduction of antibiotic resistance markers into *B. pseudomallei* and *B. mallei*. Under these guidelines the use of resistance markers in *B. pseudomallei* and *B. mallei* is limited to antibiotics not used in the treatment of humans, animals, or for agricultural purposes. Currently, the only approved antibiotic resistance markers for *B. pseudomallei* are genes encoding resistance to kanamycin (Km), zeocin (Zeo), and gentamicin (Gm). The approved antibiotic resistance markers for *B. mallei* are genes encoding resistance to Km and Zeo. Unfortunately the usefulness of these resistance markers is very limited in *B. pseudomallei* since wild type strains are intrinsically resistant to all three antibiotics (Leelarasamee and Bovornkitti, 1989; Cheng and Currie, 2005). The select-agent guidelines and the paucity of suitable selection markers have impeded the development of genetic tools for the manipulation of *B. pseudomallei*.

A second obstacle in the development of genetic tools for select-agent compliant manipulation of *B. pseudomallei* and *B. mallei* is the lack of an effective counter-selectable marker required for selection of plasmid-free clones. Common counter-selection methods utilizing tetracycline resistance and streptomycin susceptibility cannot be used in the United States under select-agent regulations, which prohibit the creation of tetracycline or streptomycin resistant *Burkholderia* strains (Reyrat et al., 1998). The non-antibiotic counter-selection marker *sacB* has been employed in *Burkholderia* with very limited success due to the frequent presence of an intrinsic *sacBC* operon in *B. pseudomallei* and *B. mallei* genomes (Chan et al., 2004; Holden et al., 2004; Essex-Lopresti et al., 2005). These limitations have greatly hampered the genetic manipulation of *B. pseudomallei* and *B. mallei*, and hindered the understanding of the biology and pathogenesis of these deadly pathogens.

Despite these impediments, progress towards the development of genetic tools for the manipulation of *Burkholderia* has been steady (DeShazer et al., 1997; Choi et al., 2006; Cuccui et al., 2007; Flannagan et al., 2008). Most importantly, select-agent compliant genetic tools for *B. pseudomallei* and *B. mallei* have recently been developed and described in two separate reports (Barrett et al., 2008; Choi et al., 2008). The first select-agent compliant genetic system was developed by Choi *et al.* and relies on the natural competency of some *B. pseudomallei* strains to linear DNA (Choi et al., 2008; Thongdee et al., 2008). This genetic system exploits the natural competency of *B. pseudomallei* to introduce linear PCR fragments carrying either Km or Zeo markers into *B. pseudomallei*. These PCR fragments integrate at desired sites in the bacterial genome and selection of transformants is achieved using 700 to 1,000 $\mu\text{g ml}^{-1}$ Km or 2,000 $\mu\text{g ml}^{-1}$ Zeo for wild-type *B. pseudomallei*. The inserted antibiotic markers can

then be excised by a site-specific recombinase expressed from a curable plasmid, leaving a scar of 86 nucleotides and creating unmarked, but not necessarily in-frame and non-polar, mutations. The second genetic system was developed by Barrett *et al.* and relies on a non-antibiotic marker that encodes tellurite resistance for the selection of *Burkholderia* transformants and a mutant *pheS* allele for counter-selection of plasmid-free clones (Barrett *et al.*, 2008).

In this report, we add to these genetic tools by describing the suicide vector pMo130 and its utility to generate in-frame and unmarked deletions in *B. pseudomallei* and *B. mallei*. As a proof of concept, we describe the deletion and complementation of the *flgK* gene in *B. pseudomallei* K96243 and 1026b strains. Additionally, we demonstrate the reliability of our system by constructing twelve unmarked deletion mutants in *B. pseudomallei* K96243 and one mutant in *B. mallei* ATCC23344.

2. Materials and methods

2.1 Bacterial strains, growth conditions, and culture media

All bacterial strains and plasmids used in this study are listed in Table 1 and 2, respectively. Manipulations of *B. pseudomallei* and *B. mallei* were performed in the BSL3 facility at UCD. Unless otherwise stated, *Burkholderia* were grown in liquid LB broth or on LB agar plates at 37°C (Fisher scientific, Hampton, NH). *E. coli* was grown at 30°C in liquid LB broth or LB agar plates, and when appropriate Km was used at 50 µg/ml for plasmid selection. For counter-selection, co-integrants were grown in YT broth, which was made by dissolving 10 grams of tryptone and 10 grams of yeast extract in 1 liter of H₂O. Sucrose was added to YT agar at a final concentration of 15% for *B. pseudomallei* and 5% for *B. mallei*. Motility was tested by stabbing LB agar plates containing 0.3% agar.

2.2 DNA methods, PCR, and Cloning

Cloning methods were performed as described previously (Sambrook, 2001). Plasmid DNA was isolated using QiaPrep Spin kit (Qiagen, Inc., Studio City, CA). Restriction enzymes and T4 DNA ligase were purchased from NEB and used as outlined by the manufacturer (New England Biolabs, Beverly, MA). Chromosomal *Burkholderia* DNA was isolated using a QIAamp DNA mini kit (Qiagen). PCR products were purified using QIAquick PCR purification kit or QIAquick gel extraction kit (Qiagen). DNA from *Burkholderia* was amplified using Advantage Taq DNA polymerase (Clontech, Palo Alto, CA) or ExTaq DNA polymerase (TAKARA, Otsu, Japan). PfuTurbo DNA polymerase was used for the amplification of DNA fragments used to construct pMo130 and pMo168 as well as site-directed mutagenesis (Stratagene, La Jolla, CA). Primers were purchased from Integrated DNA Technologies (Coralville, IA). A list of all the primers sequences used in this study is presented in supplementary Table 1S.

Touchdown PCR was performed using ExTaq or Advantage Taq DNA polymerases under the following conditions: 95°C for 5 min; 30 cycles, each consisting of 95°C for 30 sec, 66°C for 30 sec (the temperature of this step was lowered 0.3°C each cycle), and 72°C for 1 min/kb; immediately following this series, a second series of 20 cycles, consisting of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min/kb were performed. A final extension step of 72°C for 10 min was included.

2.3 Promoter prediction and cloning

Promoter prediction was done using the Neural Network Promoter Prediction website (http://www.fruitfly.org/seq_tools/promoter.html). Cloning of promoters, leader, and terminator sequences was done using repetitive PCR. Initial PCR was performed using primers

that amplify the gene of interest. The resulting PCR product was then used as a template for a second PCR using a second set of primers that overlaps the first primer set, thus introducing the desired sequence upstream or downstream from the target gene. This step was repeated until the desired promoter, leader, or terminator was introduced into the gene of interest.

The *xylE* gene was amplified by PCR to include a *tacI* promoter lacking the operator region and is referred to as *tacI** in this study (de Boer et al., 1983). The *xylE* gene was amplified by PCR using the primers XylE-F1 and XylE-R and an *algD*-pVDX18 vector as a template (Mohr et al., 1990). To introduce a *tacI** promoter to the *xylE* gene, the *xylE* PCR product was used as a template for a second PCR using the primers XylE-F2 and XylE-R. The resulting PCR product was then used for a final PCR reaction using the primers XylE-F3 and XylE-R. This final PCR product consisting of *tacI** driven *xylE* contains a *Bgl*II, *Sma*I, *Hind*III sites upstream the *xylE* gene and a *Not*I site downstream of the gene.

The modified *sacB* gene used in the construction of pMo130 was generated by PCR to include three features: the original *Bacillus subtilis* *sacB* leader signal was replaced by the presumed leader signal of the *B. mallei* *sacB* gene; the expression of the *sacB* gene was driven by a predicted *groES* promoter from *B. pseudomallei*; and the ribosomal transcriptional terminator *rrnB* T1 from *E. coli* was introduced downstream the *sacB* gene (Sarmientos et al., 1983). All three modifications were introduced into the *sacB* gene by repetitive PCR using overlapping primers. The coding sequence for the first 22 amino acids of the *B. subtilis* *sacB* gene was replaced by the coding sequence for the first 37 amino acids of the *B. mallei* *sacB* gene. The *sacB* gene was amplified from pK19mobsacB (Schäfer et al., 1994) by PCR using the primers SacB-L1F and SacB-Tr1. The resulting PCR product was then used a template for a second PCR reaction using the primers SacB-L2F and SacB-Tr2. This procedure was repeated three more times using the primers SacB-L3F, SacB-P1F, and SacB-P2F in that order as forward primers and the SacB-Tr2 as a reverse primer. This repetitive PCR resulted in the amplification of the modified *sacB* gene and was designed to include a *Spe*I and an *Eco*RI site upstream and downstream from the gene, respectively.

2.4 Construction of pMo130 and pMo168

Construction of pMo130 and pMo168 is outlined in supplementary Figure 1S. GenBank accession numbers for pMo130 and pMo168 are EU862243 and EU862244, respectively. pUC19 was used as the backbone for the construction of pMo130 and pMo168. The *Bam*HI site located in *lacZα* of pUC19 was removed by site-direct mutagenesis using primer pUC19-Δ*Bam*HI-F and pUC19-Δ*Bam*HI-R as described previously (Hamad and Nilles, 2007). The resulting pUC19-Δ*Bam*HI vector was modified by introducing the following restriction sites: *Eco*RV, *Apa*I, *Nhe*I, *Bgl*II upstream of *lacZα* and *Not*I, *Pst*I, *Bam*HI, *Xba*I, *Sph*I downstream of *lacZα* to create multiple cloning sites 1 and 2, respectively. This was accomplished by PCR amplification of the region spanning *lacZα* using primers P-MCS1-F and P-MCS2-R while the plasmid backbone was PCR amplified using P-MCS2-F and P-MCS1-R. Both PCR fragments were digested with *Bam*HI and *Nhe*I and ligated to create pMo13. Next the *xylE* amplicon containing the *tacI** promoter was digested by *Bgl*II and *Not*I and cloned into linearized pMo13 digested with the same enzymes to generate pMo13X.

To generate pMo13Xmob-km, a Km resistance cassette (*aphA*) and origin of transfer (*oriT*) were cloned into pMo13X. This was accomplished by a three-piece ligation of the pMo13X vector backbone, *oriT*, and *aphA*. The primers OriT-F and OriT-R were used to amplify the *oriT* region from pJPS8 (Sanchez-Romero et al., 1998), while the *aphA* was amplified from pK19mobsacB (Sarmientos et al., 1983) using the primers Kan-F and Kan-R. The vector backbone of pMo13Xmob-km was amplified using the primers pUC19-F and pUC19-R. The resulting *oriT* PCR product was digested using *Pac*I and *Sac*II, the *aphA* amplicon was digested with *Sac*II, and pMo13X PCR backbone was digested with *Pac*I and *Ssp*I. All three PCR

fragments were ligated to generate pMo13Xmob-km. Next a 930 bp DNA fragment was cloned as a spacer DNA between *oriT* and the *aphA* gene. This spacer DNA was amplified from pET-28b (Novagen) using the primers Spacer-F and Spacer-R. The PCR fragment was digested with *SpeI* and *SacII* and cloned into pMo13Xmob-km to create pMo130Xmob-km. The resulting pMo130Xmob-km vector has unique *SpeI*, *EcoRI* and *SacII* sites, which were used to generate pMo130 and pMo168.

To generate pMo130 the modified *sacB* gene described in the previous section was cloned into pMo130Xmob-km vector. The modified *sacB* PCR product was digested with *EcoRI* and *SacII* and cloned into pMo130Xmob-km linearized with the same enzymes to generate pMo130.

pMo168 was created as a mobilizable and replicative vector in *Burkholderia*. The replication origin, replication gene, and mobilization region used in pMo168 were PCR amplified from pSCrhaB2 (Cardona and Valvano, 2005) using the primers Rep-F and Rep-R. The resulting PCR product was digested with *NheI* and *SacII* and cloned into pMo130Xmob-km to generate pMo168.

2.5 Conjugative transfer of vectors into *Burkholderia*

Mobilizable vectors were introduced into *B. pseudomallei* and *B. mallei* by biparental mating. Briefly, cultures of *E. coli* S17-1 harboring the mobilizable vector were grown in flasks in LB broth supplemented with 50 µg/ml Km at 30°C overnight with shaking. *Burkholderia* were grown in flasks in LB broth overnight at 30°C with shaking. One ml of the donor *E. coli* and recipient *Burkholderia* were centrifuged separately and washed with 1 ml of fresh LB containing no antibiotics. The donor and recipient pellets were then suspended with 500 µl fresh LB broth and cultures were combined and filtered onto a 0.45 µm analytical filter (Nalgene, Rochester NY). The filter was placed onto LB agar containing no antibiotics and incubated for 4-8 hours at 37°C to allow for conjugation. Following incubation, the mating mixture was scraped from the filter, suspended in 3 ml of phosphate saline buffer, and 200 µl aliquots were plated by spreading onto LB agar containing Km to select for *Burkholderia* transformants and Zeo or polymyxin B (Pb) to kill donor *E. coli*.

For *B. pseudomallei*, Km was used at a concentration of 200 µg/ml while Zeo was used at 50 µg/ml. For *B. mallei*, Km was used at a concentration of 50 µg/ml for selection of *B. mallei* transformants and Pb was used at 30 µg/ml to kill donor *E. coli*. Plates were incubated for 2 days at 37°C and resulting colonies were exposed to a mist of 0.45 M pyrocatechol (Sigma-Aldrich). To prevent formation of aerosols containing *B. mallei* or *B. pseudomallei*, plates were open-faced in the BSL3 biosafety cabinet and pyrocatechol was sprayed indirectly above the plates allowing the mist to fall by gravity onto the colonies. *Burkholderia* transformants, based on yellow-colored appearance, were streaked for isolation onto LB agar containing 200 µg/ml Km and 50 µg/ml Zeo for *B. pseudomallei* and 50 µg/ml Km and 30 µg/ml Pb for *B. mallei*. Mating frequencies were calculated as the ratio of transformants over the number donor cells. The following minor notes are suggested to improve mating efficiency and recovery of transformants. Growth of donor *E. coli* and recipient *Burkholderia* should be performed in shaking flasks for increased aeration of cultures. Pb should not be used as counter-selection against donor *E. coli* in *B. pseudomallei* mating reactions, since we found that Pb increases the natural resistance of *B. pseudomallei* to Km (data not shown). Finally we would like to stress the importance of avoiding production of aerosols that contain bacteria during the pyrocatechol spraying, which should be performed indirectly as described above.

2.6 Sucrose resolution of pMo130 derivative vectors from Burkholderia co-integrants

Resolution of co-integrants was achieved by plating onto YT agar containing sucrose. Independently isolated co-integrant colonies were inoculated into 1 ml of YT broth and grown at 37°C while shaking for a minimum of 4 hours. Cultures were then serially diluted using YT broth and dilutions were plated onto YT agar containing 15% sucrose to select for resolved *B. pseudomallei* co-integrants or YT agar containing 5% sucrose to select for resolved *B. mallei* co-integrants. Resulting colonies were exposed to pyrocatechol as described above and white colonies (presumptive resolved co-integrants) were analyzed by PCR to confirm the desired deletion.

2.7 Cloning of deletion vectors and generation of Burkholderia deletion mutants

The plasmid pMo146 was used to generate an unmarked deletion in the *B. pseudomallei* *flgK* gene, *bpsl0280*, by allelic exchange in both K96243 and 1026b strains. pMo146 was constructed by cloning two PCR fragments, approximately 1 kb in length, spanning the upstream and downstream regions of the *flgK* of *B. pseudomallei* K96243. The upstream fragment was amplified using primers Δ flgK-US-NheI and Δ flgK-US-BgLII. The resulting PCR product was digested with *NheI* and *BglII*. The downstream fragment was amplified using Δ flgK-DS-BgLII and Δ flgK-DS-HindIII and the resulting PCR product was digested with *BglII* and *HindIII*. Digested upstream and downstream fragments were ligated into vector pMo130 linearized with *NheI* and *HindIII* to generate pMo146.

pMo146 was introduced into *B. pseudomallei* K96243 and 1026b strains by biparental mating as described above. Following sucrose counter-selection, plasmid-free colonies were assayed for loss of motility. Confirmation of the *B. pseudomallei* K96243 Δ flgK mutant was done by PCR using the primers FlgK-screen-F and FlgK-screen-R.

pMo176 was used to generate an unmarked deletion of the *bpss1917* gene of *B. pseudomallei* K96243 and the *bmaa0164* gene of *B. mallei* ATCC23344. The upstream region of *bpss1917* was amplified using primers Δ Bpss1917-US-NheI and Δ Bpss1917-US-BgLII and resulting PCR was digested with *NheI* and *BglII*. The downstream fragment was amplified using Δ Bpss1917-DS-BgLII and Δ Bpss1917-DS-HindIII and the resulting PCR product was digested with *BglII* and *HindIII*. Digested upstream and downstream fragments were ligated into pMo130 linearized with *NheI* and *HindIII* to generate pMo176. Deletion mutants were confirmed by PCR using the primers Δ Bpss1917-US-NheI and Δ Bpss1917-DS-HindIII.

The generation of the deletion vectors used to create the *B. pseudomallei* K96243 mutants shown in figure 5 will be described elsewhere and is available upon request.

2.8 Complementation of Δ flgK mutants of *B. pseudomallei*

pMo167 was used for the *in-cis* complementation of *B. pseudomallei* Δ flgK mutant. pMo167 was designed to insert the *flgK* gene into the large chromosome of *B. pseudomallei* K96243 downstream of the *bpsl3330* gene. pMo167 was constructed by cloning three PCR products consisting of the *flgK* gene including 210 bp upstream from the gene to retain possible ribosomal binding site, and two fragments of 1 kb in length each spanning upstream and downstream regions from the insertion site. The upstream and downstream regions were used to flank the *flgK* gene in the same orientation as the *bpsl3330* gene. A 1 kb DNA fragment downstream from the insertion region was amplified using the primers flgK-Comp-DS-SmaI and flgK-Comp-DS-Hind III. The PCR product was digested with *SmaI* and *HindIII* and cloned into pMo130 to generate pMo165. The *flgK* gene was amplified by PCR using the primers flgK-BglII and flgK-EcoRV. A 1 kb DNA fragment upstream from the insertion site was amplified by PCR using flgK-Comp-US-NheI and flgK-Comp-US-BglII. The *flgK* gene PCR product was digested with *EcoRV* and *BglII* while the upstream fragment was digested with

*Bgl*III and *Nhe*I and both fragments were ligated into pMo165 linearized with *Sma*I and *Nhe*I to create pMo167. pMo167 was introduced into the *B. pseudomallei* K96243 Δ *flgK* mutant strain by biparental mating as described above. Following sucrose resolution, plasmid-free colonies were assayed for restoration of motility. Confirmation of the *in-cis* complementation in the *B. pseudomallei* K96243 Δ *flgK* mutant was done by PCR using the primers flgK-comp-screen F and flgK-comp-screen R.

pMo173 was constructed as a replicative vector in *Burkholderia* for *in-trans* complementation of the Δ *flgK* *B. pseudomallei* mutants. The *flgK* gene was amplified by PCR to include a predicted native promoter of the *bpsl0270* gene, which is part of the flagellar operon. The *flgK* gene and the predicted promoter were generated by two repetitive PCR reactions using flgK-rep-comp-F1 and flgK-rep-comp-F2 as forward primers and flgK-comp-R as the reverse primer. The PCR product was then digested with *Nhe*I and *Hind*III and ligated with pMo168 to generate pMo173. pMo173 was then introduced into the Δ *flgK* mutants of *B. pseudomallei* K96243 and 1026b strains by biparental mating.

3. Results

3.1 Features of the select-agent compliant vectors pMo130 and pMo168

The maps of the select-agent compliant plasmids pMo130 and pMo168 are presented in Figure 1A and their construction is depicted in supplementary Figure 1S. pMo130 carries a ColE1 origin of replication derived from pUC19 plasmid and is a suicide vector in *Burkholderia*. This suicide vector can be used for allelic exchange to generate in-frame deletions or insertions into the genomes of *Burkholderia*. pMo168 carries the pBBR1 origin of replication and can be maintained extra-chromosomally in *Burkholderia*. The replicative vector pMo168 can be used for *in-trans* complementation or gene expression in *Burkholderia*. pMo130 and pMo168 carry an origin of transfer, which allows for their mobilization into *Burkholderia* through conjugation. Both pMo130 and pMo168 carry the reporter gene *xylE* which encodes a catechol-2,3-dioxygenase, an enzyme that turns colorless catechol substrate into bright yellow-colored 2-hydroxymuconic semialdehyde (Zukowski et al., 1983; Lee et al., 1996). *xylE* is expressed from a *tacI* promoter lacking the operator region which allows for constitutive and high level of expression in *Burkholderia*. Flanking *xylE* are two multiple cloning sites, MCS1 and MCS2, which contain unique restriction sites that can be used for cloning. Selection for pMo130 and pMo168 is achieved by the activity of an *aphA* gene, which confers Km resistance and is an approved antibiotic selection marker for *B. pseudomallei* and *B. mallei*. Finally, counter-selection against pMo130 is achieved through a modified *sacB* gene optimized for *Burkholderia*. The modified *B. subtilis* *sacB* gene has the leader sequence of the *B. mallei* *sacB* and is expressed from a predicted promoter of the *groES* genes of *B. pseudomallei*.

3.2 Use of pMo130 to create an unmarked flagellar *B. pseudomallei* mutant

To demonstrate the utility of pMo130 for allelic exchange, the *bpsl0280* gene of *B. pseudomallei* encoding the flagellar gene *flgK* was targeted for deletion. One kb upstream and downstream fragments flanking the *bpsl0280* gene of *B. pseudomallei* K96243 were cloned into pMo130. The resulting plasmid, pMo146, was conjugated into *B. pseudomallei* K96243 and 1026b to isolate co-integrants representing the first crossover event required for allelic exchange (Fig. 1B). Selection and identification of *B. pseudomallei* co-integrants was achieved using a combination of Km selection and visual detection of Xyle activity following catechol treatment (Fig. 2A). Wild-type *B. pseudomallei* is resistant to Km mainly through the activity of efflux pumps (Moore et al., 1999; Choi et al., 2008). However we found that plating of wild type *B. pseudomallei* on LB agar plates supplemented with 200 μ g/ml Km results in a reduction of 7.3 log₁₀ and 6.2 log₁₀ in colony counts for the K96243 and 1026b strains, respectively. To overcome this leaky selection capacity of Km, colonies that grew on Km agar plates were

sprayed with catechol to detect XylE activity. The activity of XylE in *Burkholderia* turns co-integrant colonies into bright yellow color while spontaneously Km resistant colonies remain white in color (Fig. 2A-C). Typically 30-50% of *B. pseudomallei* colonies recovered on Km agar plates were true transformants (Fig. 2A), and the efficiency of co-integrants formation was 2.3×10^{-7} and 5.6×10^{-7} per donor cell for the K96243 and 1026b strains, respectively.

The resolution of pMo146 co-integrants was achieved through sucrose counter-selection which is required for the second crossover event and vector loss (Fig. 1B). The plating efficiency of *B. pseudomallei* containing a single copy of pMo146 on YT agar containing 15% sucrose vs. LB agar was approximately 1% (Fig. 2D). In contrast, plasmid-free *B. pseudomallei* grew equally well on YT agar containing 15% sucrose and on LB agar (Fig. 2D). Because counter-selection using 15% sucrose does not inhibit growth of plasmid-free *B. pseudomallei*, it is unlikely to cause selection for unintended secondary mutations. To determine the efficiency of sucrose resolution, pMo146 co-integrants were plated onto LB plates or 15% sucrose YT agar and resolution was determined visually through XylE activity. None of the colonies from the LB plates had lost the integrated vector; however, all of the colonies that grew on the sucrose agar plate were plasmid-free as determined by the loss of XylE activity (Fig. 2E). Experiments utilizing pMo146 and other pMo130 derivatives show that during sucrose resolution, greater than 99% of resulting colonies are plasmid free for both K96243 and 1026b strains of *B. pseudomallei*.

Screening for deletion mutants is typically performed by PCR of resolved colonies. However, since this flagellar deletion has a known phenotype, the *flgK* deletion mutant was detected by its loss-of-motility phenotype. Of the twenty resolved colonies screened from each strain, thirteen colonies of the K96243 strain and eight colonies of the 1026b strain were found to be non motile. The motility of the wild type and the $\Delta flgK$ mutants in the 1026b and K96243 strains are shown in Figure 3.

3.3 Complementation of the *B. pseudomallei* flagellar mutants

Complementation of the flagellar *B. pseudomallei* mutants was done both *in-cis* and *in-trans* to demonstrate that the mutation was non-polar, and that pMo130 can be reused for allelic exchange in the same mutant strain. For *in-cis* complementation, pMo130 was used to construct an unmarked insertion of *flgK* into the large chromosome of the $\Delta flgK$ mutant of *B. pseudomallei* K96243. The *flgK* gene, *bpsl0280*, was inserted by allelic exchange downstream and in the same orientation as the *bpsl3330* gene. *flgK* was cloned between the desired insertion site into pMo130 and the resulting vector was introduced into the $\Delta flgK$ mutant of *B. pseudomallei* K96243 by conjugation. Following sucrose counter-selection, resolved colonies were screened for complementation by the restoration of motility. Nine out of the twenty resolved colonies tested were found to be motile. Motility of the *B. pseudomallei* K96243 $\Delta flgK$ mutant complemented through the insertion of *flgK* downstream from the *bpsl3330* is shown in figure 3A. PCR was then used to confirm that the *flgK* gene “*bpsl0280*” was inserted downstream of the *bpsl3330* gene and not at the homologous DNA sequence of the *bpsl0280* deletion allele. PCR screening using primers flanking of the *flgK* gene confirmed the deletion of *flgK* (Fig. 4, lanes 1- 2) and demonstrated that the *flgK* gene did not reinsert at the homologous deletion allele in the complemented *flgK* mutant (Fig. 4, lanes 1-3). PCR screening of the complemented mutant using primers flanking the insertion region confirmed that the *flgK* gene was indeed inserted at the desired region downstream of *bpsl3330* (Fig. 4, lanes 4-6). Taken together, these results demonstrate that pMo130 is suitable for genomic complementation in *B. pseudomallei* and that this vector can be reused to introduce multiple mutations or insertions into the same strain.

In-trans complementation was achieved by cloning the *flgK* gene into the replicative vector pMo168 to generate pMo173. pMo168 and pMo173 were then introduced into the $\Delta flgK$

mutant strains of *B. pseudomallei* by conjugation and transformants were recovered using Km selection and detection of Xyle activity. Complementation of the non-motile $\Delta flgK$ mutants through the introduction of pMo173 restored motility in both mutant strains, while the un-complemented mutant or the mutant carrying empty vector remained non-motile (Fig. 3B-C). These results demonstrate the utility of pMo168 for *in-trans* complementation and gene expression in *Burkholderia*.

3.4 Construction of twelve *B. pseudomallei* mutants and a *B. mallei* mutant

To further demonstrate the reliability of pMo130 in generating unmarked deletions in *B. pseudomallei*, we generated numerous other deletions in the K96243 strain. The K96243 strain, rather than strain 1026b, was used for constructing these mutations because the genomic sequence of the K96243 is available. Twelve unmarked mutants were constructed by cloning the appropriate flanking DNA fragments into pMo130 and using the resulting vectors for allelic exchange as described above for the flagellar deletion. PCR confirmation of the twelve mutants is shown in figure 5 (Fig. 5 lanes 2-23). These mutants included deletions of *bpss1161-1162*, *bpss2345*, *bpss1160-1161*, *bpsl0074-0075*, *bpss1359-1360*, and *bpss2314* which encode putative response regulators and/or sensor kinases; *bpss0031*, *bpss1917*, *bpss1803* which encode putative transcriptional regulators; *bpss1957* which encodes a putative 6-phosphofructokinase; and *bpss0542* and *bpss0542-0543* which encode putative *sacC* and *sacBC* genes, respectively. The transformation and resolution efficiencies obtained during the creation of these mutants were similar to those observed for the *flgK* deletion. The rate of mutant recovery differed for each deletion, and generally 20-50% of the screened resolved colonies contained the deleted form as confirmed by PCR.

The use of pMo130 for generating deletions in *B. mallei* ATCC23344 strain was also evaluated. The *bmaa00164* gene of *B. mallei* is homologous to *bpss1917* of *B. pseudomallei* K96243. pMo176, which was used to delete *bpss1917* in *B. pseudomallei* K96243, was used to delete *bmaa00164* in *B. mallei* through allelic exchange. pMo176 was introduced into *B. mallei* by conjugation and co-integrants were selected using 50 µg/ml Km since *B. mallei* is sensitive to Km. The efficiency of co-integrants formation was 2.3×10^{-7} per donor cell, and all of the recovered Km resistant colonies were true co-integrants as determined by Xyle activity. *B. mallei* co-integrants were plated onto YT agar plus 5% sucrose to allow for the resolution of the integrated pMo176. The resolution rate of *B. mallei* co-integrants was lower than that of *B. pseudomallei* and only 10-20% of the colonies that grew on sucrose plates were plasmid-free as determined by loss of Xyle activity. Resolved colonies were screened by PCR and five out of the sixteen analyzed colonies were found to contain the mutation. PCR of the wild type *B. mallei* gene *bmaa00164* as well as its deletion allele are shown in figure 5 (Fig. 5 lanes 24-25).

4. Discussion

Genetic manipulation of *B. pseudomallei* and *B. mallei* has been difficult and their classification as category B select agents has greatly limited the development of new and useful genetic tools. The vectors presented in this study add to the repertoire of available tools that allow for the select-agent compliant manipulation of *B. pseudomallei* and *B. mallei*. Our system overcomes some of the limitations of other systems, including the recovery of false positive transformants despite the use of high antibiotic concentrations for selection, lack of natural competence of specific *Burkholderia* strains for DNA uptake, and generation of DNA scars following marker excision (Choi et al., 2008). Additionally, our system uses homologous recombination to exchange mutant alleles for wild type alleles (or vice-versa) in the genome of *Burkholderia*. Our genetic system is reliable for generating deletion mutants in *B. pseudomallei*, and we have successfully utilized this system to generate numerous in-frame and unmarked deletions both

in *B. pseudomallei* K96243 and 1026b (this work; M. Hamad and S. Zajdowicz unpublished). We have also demonstrated the usefulness of this system to produce mutations in *B. mallei* ATCC23344 (this work; and S. Zajdowicz). A minor drawback in the manipulation of *B. mallei* is that sucrose counter-selection generates a lower proportion number of resolved colonies than in *B. pseudomallei* and can lead to recovery of spontaneous deletions of the endogenous *sacB* region of *B. mallei* (DeShazer et al., 2001). However, these drawbacks can be overcome by using our allelic exchange vector in a $\Delta sacB$ *B. mallei* background. A $\Delta sacB$ *B. mallei* background strain has been created and is fully virulent in the mouse model of glanders infection (DeShazer et al., 2001; Schell et al., 2008).

In conclusion, the genetic tools described herein are a valuable addition to the repertoire of *Burkholderia* genetic systems and will help to advance our understanding of the biology and pathogenesis of these dangerous pathogens.

Supplementary-material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Dr. Mike Schurr for providing *algD*-pVDX18 vector, Dr. Miguel Valvano for providing pSCrhaB2 vector, and Dr. Victor De Lorenzo for providing pJPS8 vector. This research was supported Rocky Mountain Regional Center of Excellence grant AI-065357 awarded to MIV and NIH R21 grant AI074606-01 awarded to RKH.

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Abbreviations

Pb	Polymyxin B
Gm	gentamicin
Km	kanamycin
Zeo	zeocin

Figure 1.

Plasmid maps and allelic exchange. A. Plasmid maps of pMo130 and pMo168. pMo130 and pMo168 carry a kanamycin resistance cassette (Km^R) and the reporter gene *xylE*. pMo130 carries a ColE1 origin of replication (*ori*) and an RK2 origin of transfer (*oriT*) and is a mobilizable and suicide vector in *Burkholderia*. Multiple cloning sites for pMo130 are MCS-1: *ApaI*, *NheI*, *BglIII*, *SmaI*, *HindIII* and MCS-2: *NotI*, *PstI*, *BamHI*, *XbaI*, *SphI*. pMo168 is a mobilizable vector derived from the vector pSCRhaB2 and is a replicative vector in *Burkholderia*. pMo168 carries the mobilization region (*mob*) and replication features of pBBR1. Multiple cloning sites for pMo168 are MCS-1: *ApaI*, *NheI*, *BglIII*, *SmaI*, *HindIII* and MCS-2: *PstI*, *BamHI*, *XbaI*, *SphI*. B. The use of allelic exchange to construct a *flgK* deletion

in *B. pseudomallei*. DNA fragments upstream and downstream the *flgK* gene are cloned into pMo130 to generate pMo146. pMo146 is introduced into *B. pseudomallei* through conjugation and co-integrant are recovered by kanamycin selection and screening for *xylE* positive yellow colonies. Resolved co-integrant are recovered by sucrose counter-selection and screening for *xylE* negative white colonies. Colonies are analyzed by PCR or phenotypic analysis to distinguish deletion mutants from wild type isolates.

Figure 2.

Utility of a pMo130 derivative vector in *Burkholderia*. A. Use of *xylE* of pMo130 for detection of *Burkholderia* transformants. A 200 μ l of conjugation reaction between wild type *B. pseudomallei* K96243 and donor *E. coli* to introduce the pMo130 derivative, pMo146, were plated on Km-Zeo LB agar. Following 2 days incubation, plates were indirectly sprayed with catechol to visualize yellow-colored transformants and to distinguish them from white-colored wild type nontransformant colonies. B. Pure culture of *B. pseudomallei* K96243 and *B. pseudomallei* K96243 carrying a single copy of pMo146 integrated in the large chromosome prior to catechol treatment. C. Same as B after catechol treatment. D. Use of the modified *sacB* of pMo130 for killing of *B. pseudomallei* K96243 carrying a single copy of pMo146 integrated in the large chromosome. Wild type *B. pseudomallei* K96243 and pMo146 co-integrant were serially diluted at 1:10 and plated on LB agar or YT agar plus 15% sucrose, and colonies were scored 2 days post incubation. E. Use of the modified *sacB* for counter-selection of plasmid-free resolvants of *B. pseudomallei* K96243. pMo146 co-integrant culture were plated onto LB agar and YT agar plus 15% sucrose and incubated for 2 days. Resulting colonies were sprayed with catechol to determine resolution.

Figure 3.

Motility plate assays. A. Motility of wild type *B. pseudomallei* K96243 (Wt), its $\Delta flgK$ derivative mutant ($\Delta flgK$), and the *in-cis* $\Delta flgK$ complemented mutant ($\Delta flgK$ comp). B. Motility of wild type *B. pseudomallei* K96243, its $\Delta flgK$ derivative mutant carrying empty replicative vector pMo168, and its $\Delta flgK$ derivative mutant carrying the complementation vector pMo173. C. Motility of wild type *B. pseudomallei* 1026b, its $\Delta flgK$ derivative mutant, and the $\Delta flgK$ mutant complemented *in-trans* with pMo173.

Figure 4.

PCR confirmation of *B. pseudomallei* K96243 $\Delta flgK$ mutant and the *in-cis* complemented derivative ($\Delta flgK$ comp). PCR confirmation of the *flgK* deletion was performed using the outside primers flgK-screen-F and flgK-screen-R. PCR confirmation of the insertion of *flgK* downstream *bpsl3330* was performed using the primers flgK-comp-screen-F and flgK-comp-screen-R which are outside the complementation region. The $\Delta flgK$ mutant had a 2 kb deletion in comparison to wild-type *B. pseudomallei* K96243. The complemented strain had a 2 kb insertion in comparison to wild-type or un-complemented $\Delta flgK$ mutant.

Figure 5.

PCR confirmation of 12 unmarked deletions in *B. pseudomallei* K96243 (lanes 2-23), and one unmarked deletion in *B. mallei* ATCC23344 (lanes 24-25). L: DNA ladder. Wt and M are PCR performed on wild type and mutant strains, respectively. Lane 1-2: PCR confirmation of $\Delta bpss1917$, wild type allele is 2666 bp and mutant allele is 2000 bp. Lane 3-4: PCR confirmation of $\Delta bpss1161-1162$, wild type allele is 1257 bp and mutant allele is 303 bp. Lane 5-6: PCR confirmation of $\Delta bpss1803$, wild type allele is 2570 bp and mutant allele is 2000 bp. Lane 7-8: PCR confirmation of $\Delta bpss2345$, wild type allele is 2175 bp and mutant allele is 229 bp. Lane 9-10: PCR confirmation of $\Delta bpss1160-1161$, wild type allele is 3102 bp and mutant allele is 800 bp. Lane 11-13: PCR confirmation of $\Delta bpss0542$ and $\Delta bpss0542-0543$ wild type

allele is 5393 bp, $\Delta bpss0542$, M1, is 3758 bp, and $\Delta bpss0542-0543$, M2, is 2090 bp. Lane 14-15: PCR confirmation of $\Delta bpss0031$, wild type allele is 2582 bp and mutant allele is 2000 bp. Lane 16-17: PCR confirmation of $\Delta bpss1957$, wild type allele is 1394 bp and mutant allele is 641 bp. Lane 18-19: PCR confirmation of $\Delta bpsl0074-0075$, wild type allele is 3151 bp and mutant allele is 1165 bp. Lane 20-21: PCR confirmation of $\Delta bpss1359-1360$, wild type allele is 2071 bp and mutant allele is 284 bp. Lane 22-23: PCR confirmation of $\Delta bpss2314$, wild type allele is 2017 bp and mutant allele is 284 bp. Lane 24-25: PCR confirmation of *bmaa0164* deletion in *B. mallei* ATCC23344, wild type allele is 2666 bp and mutant allele is 2000 bp.

Table 1

Bacterial strains.

Strains	Relevant features	Reference
<i>B. pseudomallei</i> K96243	Human Clinical isolate; Km ^R Gm ^R Zeo ^R Pb ^R	(Holden et al.,
<i>B. pseudomallei</i> 1026b	Human Clinical isolate; Km ^R Gm ^R Zeo ^R Pb ^R	(DeShazer et al.,
<i>B. mallei</i> ATCC23344	Human Clinical isolate; Km ^S Gm ^S Zeo ^S Pb ^R	(Yabuuchi et al.,
<i>B. pseudomallei</i> K96243 <i>flgK</i> ::pMo146	<i>B. pseudomallei</i> K96243 with pMo146 inserted into <i>bpsl0280</i> , <i>xylE</i> +	This study
<i>B. pseudomallei</i> 1026b <i>flgK</i> ::pMo146	<i>B. pseudomallei</i> 1026b with pMo146 inserted into <i>bpsl0280</i> , <i>xylE</i> +	This study
<i>B. pseudomallei</i> K96243 Δ <i>flgK</i>	Unmarked deletion of <i>bpsl0280</i> , nonmotile	This study
<i>B. pseudomallei</i> 1026b Δ <i>flgK</i>	Unmarked deletion of <i>bpsl0280</i> , nonmotile	This study
<i>B. pseudomallei</i> K96243 Δ <i>flgK</i> comp	Unmarked deletion of <i>bpsl0280</i> , complemented by an unmarked insertion of <i>bpsl0280</i> downstream of <i>bpsl3330</i> , motile	This study
<i>B. pseudomallei</i> K96243 Δ <i>flgK</i> + pMo168	Unmarked deletion of <i>bpsl0280</i> carrying empty replicative vector pMo168, nonmotile	This study
<i>B. pseudomallei</i> K96243 Δ <i>flgK</i> + pMo173	Unmarked deletion of <i>bpsl0280</i> carrying complementation replicative vector pMo173, motile	This study
<i>B. pseudomallei</i> 1026b Δ <i>flgK</i> + pMo173	Unmarked deletion of <i>bpsl0280</i> carrying complementation replicative vector pMo173, motile	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1160-1161</i>	Unmarked deletion of <i>bpss1160-1161</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1161-1162</i>	Unmarked deletion of <i>bpss1161-1162</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss10074-0075</i>	Unmarked deletion of <i>bpss10074-0075</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1359-1360</i>	Unmarked deletion of <i>bpss1359-1360</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss2314</i>	Unmarked deletion of <i>bpss2314</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss2345</i>	Unmarked deletion of <i>bpss2345</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss0031</i>	Unmarked deletion of <i>bpss0031</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1957</i>	Unmarked deletion of <i>bpss1957</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1917</i>	Unmarked deletion of <i>bpss1917</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss0542</i>	Unmarked deletion of <i>bpss0542</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss1803</i>	Unmarked deletion of <i>bpss1803</i>	This study
<i>B. pseudomallei</i> K96243 Δ <i>bpss0542-0543</i>	Unmarked deletion of <i>bpss0542-0543</i>	This study
<i>B. mallei</i> ATCC23344 Δ <i>bmaa0164</i>	Unmarked deletion of <i>bmaa0164</i>	This study
<i>E. coli</i> DH5 α	Cloning strain, F- f80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA</i> -argF)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (rk-, mk+)	Laboratory stock
<i>E. coli</i> JM109	Cloning strain, e14-(McrA-) <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> (rk- mk+) <i>supE44</i> <i>relA1</i> Δ (<i>lac</i> -	Stratagene
<i>E. coli</i> S17.1	For conjugal transfer, <i>recA</i> <i>thi</i> <i>pro</i> <i>hsdRM</i> + RP4:2-Tc:Mu:KmTn7 TpR SmR	(R. Simon, 1983)

Pb: polymyxin B, Km: Kanamycin, Gm: Gentamicin, Zeo: zeocin. R: resistance, S: susceptibility.

Table 2

Plasmids.

Plasmids	Relevant features and use	Reference
pET-28b	Source of spacer DNA	Novagen
algD-pVDX18	Source of <i>xylE</i>	(Mohr et al., 1990)
pJPS8	Source of RK2 <i>oriT</i>	(Sanchez-Romero et al., 1998)
pSCrhaB2	Source of <i>oripBBR1</i> and <i>mob</i> region	(Cardona and Valvano, 2005)
pK19 <i>mobsacB</i>	Source of <i>sacB</i> and <i>aphA</i>	(Schafer et al., 1994)
pUC19	Vector back bone, source of ColE1 <i>ori</i>	(Yanisch-Perron et al., 1985)
pMo13	pUC19 with MCS-1 and MCS-2	This study
pMo13X	pMo13 plus <i>xylE</i>	This study
pMo13Xmob-km	pMo13X plus RK2 <i>oriT</i> and <i>aphA</i> , Km ^R	This study
pMo130Xmob-km	pMo13Xmob-km plus spacer DNA	This study
pMo130	Suicide vector for allelic exchange in <i>Burkholderia</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	This study
pMo146	Suicide vector used to construct <i>B. pseudomallei</i> K96243 Δ <i>flgK</i> and <i>B. pseudomallei</i> 1026b Δ <i>flgK</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	This study
pMo168	Replicative vector for <i>Burkholderia</i> ; <i>oripBBR1</i> , <i>mob+</i> , <i>xylE</i> , Km ^R	This study
pMo167	Suicide vector used for <i>in-cis</i> complementation of <i>B. pseudomallei</i> K96243 Δ <i>flgK</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>flgK</i> , <i>sacB</i> , Km ^R	This study
pMo173	pMo168 plus <i>flgK</i> used for <i>in-trans</i> complementation of <i>B. pseudomallei</i> Δ <i>flgK</i> ; <i>oripBBR1</i> , <i>mob+</i> , <i>flgK</i> , <i>xylE</i> , Km ^R	This study
pMo176	Suicide vector used to construct <i>B. pseudomallei</i> K96243 Δ <i>bps1917</i> and <i>B. mallei</i> ATCC23344 Δ <i>bmaa0164</i> ; ColE1 <i>ori</i> , RK2 <i>oriT</i> , <i>xylE</i> , <i>sacB</i> , Km ^R	This study