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Versatile Nourseothricin and Streptomycin/Spectinomycin Resistance Gene Cassettes and Their Use in Chromosome Integration Vectors

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Abstract

An obstacle for the development of genetic systems for many bacteria is the limited number of antibiotic selection markers, especially for bacteria that are intrinsically antibiotic resistance or where utilization of such markers is strictly regulated. Here we describe the development of versatile cassettes containing nourseothricin, streptomycin/spectinomycin, and spectinomycin selection markers. The antibiotic resistance genes contained on these cassettes are flanked by *loxP* sites with allow their *in vivo* excision from the chromosome of target bacteria using Cre recombinase. The respective selection marker cassettes were used to derive mini-Tn7 elements that can be used for single-copy insertion of genes into bacterial chromosomes. The utility of the selection markers was tested by insertion of the resulting mini-Tn7 elements into the genomes of *Burkholderia thailandensis* and *B. pseudomallei* efflux pump mutants susceptible to aminoglycosides, aminocyclitols, and streptothricins, followed by Cre-mediated antibiotic resistance marker excision. The versatile nourseothricin, streptomycin/spectinomycin and spectinomycin resistance *loxP* cassette vectors described here extend the repertoire of antibiotic selection markers for genetic manipulation of diverse bacteria that are susceptible to aminoglycosides and aminocyclitols.

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Keywords

antibiotics; selection; nourseothricin; spectinomycin; streptomycin; cassette; mini-Tn7

1. Introduction

Genetic manipulation of bacteria heavily relies on availability of antibiotic resistance selection markers. Replicative plasmids contain selection markers for their initial establishment and maintenance in the bacterial cell (Schweizer, 2008). Similarly, random or site-specific chromosomal insertion of transposons requires an initial selection step, but then replicate along with the chromosome and require no continued selection for stable maintenance. Lastly, generation of chromosomal gene knockouts generally involves homologous recombination of a selectable marker, usually an antibiotic resistance cassette, into the targeted chromosomal region. The issue with many bacteria is that they are intrinsically antibiotic resistant and the repertoire of available selection markers is therefore slim. An additional hindrance for researchers studying Select Agent bacteria in the United States is that the use of antibiotic resistance selection markers is highly regulated at the Federal level by the Select Agent Program (Schweizer and Peacock, 2008). These issues can be addressed by recycling the few workable and approved antibiotic resistance markers using a site-specific recombinase or developing additional selection markers.

Nourseothricin is a broad-spectrum streptothricin aminoglycoside antibiotic. It has previously seen limited use as antibiotic growth promoter in pigs (Hummel et al., 1986; Teillant et al., 2015), but is no longer used in human and veterinary medicine due to nephrotoxicity (Krugel et al., 1993; Maier et al., 2006). Nourseothricin resistance (Nat^r) is mediated by an *N*-acetyltransferase encoded by the *nat* gene of *Streptomyces noursei* (Krugel et al., 1993). The Nat^r marker has been used for genetic manipulation of yeast (*Saccharomyces cerevisiae*) (Goldstein and McCusker, 1999) and bacteria, including *B. pseudomallei* (Choi et al., 2008) and *Francisella tularensis* (Maier et al., 2006). In contrast to Nat^r, streptomycin/spectinomycin resistance (Sm^r/Spc^r) markers have been widely used in bacterial genetics, especially the Ω Sm^r/Spc^r cassette contained on pHP45 Ω (Prentki and Krisch, 1984). The Sm^r/Spc^r determinant contained on this plasmid is an *O*-adenyltransferase encoded by the *aadA* gene. This enzyme adenylates streptomycin at the 3-OH of its glucosamine ring and spectinomycin at the 9-OH position of its actinamine ring (Yamada et al., 1968). Because we were mostly interested in exploiting the Spc^r conferred by AadA, we only used Spc^r selection in our experiments. Unlike AadA which adenylates both streptomycin and spectinomycin, Aad9 adenytransferase encoded by the *aad9* gene from *Enterococcus faecalis* only modifies spectinomycin (Leblanc et al., 1991). The *aad9* gene has been mostly used as selection marker in Gram-positive bacteria (Sreenivasan and Fives-Taylor, 1994; Charpentier et al., 2004). Its availability extends the utility of tools containing the spectinomycin selection marker to bacteria where streptomycin resistance selection cannot be used either because of intrinsic resistance due to ribosomal mutations or regulatory restrictions, e.g. the select agent *B. mallei*. *B. mallei* is closely related to *B. pseudomallei* and of considerable interest because of its biothreat potential (Dance, 2005).

2. Materials and methods

2.1 Bacterial strains, media and growth conditions

E. coli strains used in this study were DH5 α (Liss, 1987) and RHO3 (Lopez et al., 2009). *Burkholderia* strains used were *B. thailandensis* E264 (Brett et al., 1998) and its (*amrRAB-oprA*) efflux pump mutant derivative DW503 (Burtnick et al., 2001), and *B. pseudomallei* strains 1026b (DeShazer et al., 1997) and (*amrRAB-oprA*) efflux pump mutant derivative Bp50 (Choi et al., 2008), and Bp82 (Propst et al., 2010) and its (*amrAB-oprA*) efflux pump mutant derivative Bp82.27 (laboratory isolate). All experiments with virulent *B. pseudomallei* strains 1026b and Bp50 were performed at Colorado State University at BSL-3 using Select Agent certified laboratory facilities and compliant methods. Bp82 and its (*amrAB-oprA*) derivative Bp82.27 were grown in media supplemented with 80 μ g/ml adenine. Bp82 is excluded from Select Agent regulations (www.selectagents.gov/SelectAgentsandToxinsExclusions.html). All experiments with strain Bp82 and its derivatives were conducted at BSL-2 with Institutional Biosafety Committee approval. Bacteria were routinely grown at 37°C in Lennox Luria broth (LB) (Sezonov et al., 2007) or on LB agar purchased from MO BIO Laboratories, Carlsbad, CA. *E. coli* RHO3 is a *asd* mutant and requires 400 μ g/ml diaminopimelic acid (DAP; LL-, DD-, and meso-isomers; Sigma, St. Louis, MO) for growth on rich media (Lopez et al., 2009). Strains containing temperature-sensitive (TS) plasmid derivatives or TS alleles were grown at 30°C (permissive temperature) or 42°C (non-permissive temperature). Antibiotics were added at the following concentrations: 100 μ g/ml ampicillin, 32 μ g/ml nourseothricin and 100 μ g/ml spectinomycin for *E. coli*; 32 μ g/ml nourseothricin and 100 μ g/ml spectinomycin for *B. thailandensis* and *B. pseudomallei* (*amrAB-oprA*) strains. Minimal inhibitory concentrations (MICs) were determined in Mueller-Hinton broth (Becton Dickinson and Company, Sparks, MD) by the 2-fold broth microdilution technique following Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute, 2015). The MICs were recorded after incubation at 37°C for 24 h. Antibiotics were purchased from Sigma, except nourseothricin, which was obtained from Jena Bioscience, Jena, Germany.

2.2 Plasmids

Previously constructed plasmids used in this study include pCR2.1 (ThermoFisher Scientific, Waltham, MA), pLOX1 (Choi et al., 2008), pTNS3 (Choi et al., 2008) and pUC18T-mini-Tn 7T (Choi and Schweizer, 2006). The GenBank accession numbers for these plasmids are EU215436 for pLOX1; EU215432 for pTNS3; and AY599230 for pUC18T-mini-Tn 7T.

2.3. Construction of pLNT1, pLSP1 and pLSP2

Plasmid pLNT1 was constructed in several steps. First, the *nat* gene and its predicted promoter (www.fruitfly.org/seq_tools/promoter.html) were PCR amplified from pAG36 (Goldstein and McCusker, 1999) using primers natB (5'-CCAGATCTGTTTAGCTTGCCTTGTC) and RBS Reverse (5'-TAACTTCGCATCTGGGCAGATGATG). The resulting 1,109-bp PCR fragment was gel purified (GeneJET Gel Extraction Kit, ThermoFisher Scientific) and ligated into the TA cloning vector pCR2.1 to yield pCR2.1-*nat*. Second, the *nat* gene and its predicted promoter

were excised from pCR2.1-*nat* on a 1,129-bp *EcoRI* fragment which was gel-purified and then ligated into the *EcoRI* site of pLOX1 to form pLNT1.

For construction of pLSP1, the *aadA* gene and its predicted promoter were PCR amplified from pHP45Ω (Prentki and Krisch, 1984; Prentki et al., 1991) on a 1,163-bp fragment using primer set Sm^r/Spe^r-UP (5'- CGAACCCAGTGGACATAAGCC) and Sm^r/Spe^r-DN (5'- CCTGATAGTTTGGCTGTCAGC). This fragment was gel purified and cloned into pCR2.1 to yield pCR2.1-*aadA*. Next, a 1,183-bp *EcoRI* fragment carrying *aadA* and its predicted promoter was excised from pCR2.1-*aadA* and after gel purification cloned into pLOX1 to yield pLSP1.

A 916-bp DNA fragment containing the *aad9* gene was designed and synthesis conducted by GenScript, Piscataway, NJ. This fragment contains the previously published sequence (Leblanc et al., 1991)(GenBank accession number M69221.1) with several changes, including an ATG instead of the native TTG start codon and the chloramphenicol acetyl transferase (*cat*) gene promoter (Le Grice et al., 1982) instead of the native promoter. The *aad9* native ribosome-binding site and transcription termination signals were maintained. The 916-bp *EcoRI* fragment was cloned into pUC57 (GenScript) to form pPS2729. Next, the *aad9* gene and the *cat* promoter were excised from pPS2729 on a 916-bp *EcoRI* fragment, which was gel-purified and then ligated into the *EcoRI* site of pLOX1 to form pLSP2.

The GenBank accession numbers for pLNT1, pLSP1 and pLSP2 are KU936034, KU936035, and KU936036, respectively.

2.4. Construction of mini-Tn7T-*nat*, mini-Tn7T-*aadA* and mini-Tn7T-*aad9*

For construction of mini-Tn7T-*nat*, a 1,322-bp *XbaI* fragment blunt-ended with T4 DNA polymerase and containing *loxP-nat-loxP* from pLNT1 was subcloned into the *EcoRV* site of pUC18T-mini-Tn7T and a derivative in which the *nat* gene was oriented towards the mini-Tn7 multiple cloning site was retained. Similarly, mini-Tn7T-*aadA* and mini-Tn7T-*aad9* were obtained by subcloning a 1,350-bp *SmaI* fragment containing *loxP-aadA-loxP* from pLSP1 or a 1,081-bp *SmaI* fragment containing *loxP-aad9-loxP* from pLSP2 into the *EcoRV* site of pUC18T-mini-Tn7T, and retaining isolates in which *aadA* or *aad9* were oriented opposite to the mini-Tn7 multiple cloning site.

The GenBank accession numbers for pUC18T-mini-Tn7T-*nat*, pUC18T-mini-Tn7T-*aadA* and pUC18T-mini-Tn7T-*aad9* are KU936037, KU936038 and KU936039, respectively.

2.5. Transposition of mini-Tn7T-*nat*, mini-Tn7T-*aadA* and mini-Tn7T-*aad9* into *B. thailandensis* and *B. pseudomallei*

The helper plasmid pTNS3 (Choi et al., 2008) and the mini-Tn7 delivery vectors pUC18T-mini-Tn7T-*nat*, pUC18T-mini-Tn7T-*aadA* or pUC18T-mini-Tn7T-*aad9* were transformed into *E. coli* mobilizer strain RHO3 (Lopez et al., 2009). Co-conjugation of both helper and delivery plasmids into *B. thailandensis* DW503 and *B. pseudomallei* strains Bp50 and Bp82.27 was achieved by biparental mating using a previously described method (Lopez et al., 2009). Nat^r and Spc^r transformants were selected at 37°C on LB-agar plates with either 32–50 µg/ml nourseothricin or 100 µg/ml spectinomycin. Mini-Tn7 insertions at the

predicted Tn7 insertion sites, two in *B. thailandensis* (Choi et al., 2005) and three in *B. pseudomallei* (Choi et al., 2008; Bruckbauer et al., 2015), were detected by PCR employing previously published methods (Choi et al., 2005; Choi et al., 2008; Bruckbauer et al., 2015).

2.6. Cre-mediated excision of antibiotic selection markers and curing of Cre expression plasmid

Cre-mediated excision of chromosomally-inserted nourseothricin and spectinomycin resistance markers was achieved using previously described procedures and pCRE5 (Choi et al., 2008). Briefly, using a rapid transformation procedure (Choi et al., 2008) pCRE5 was transformed into *B. thailandensis* DW503 or *B. pseudomallei* Bp50 or Bp82.27 isolates containing either a chromosomally-integrated mini-Tn7-*nat*, mini-Tn7-*aadA* or mini-Tn7-*aad9*, and kanamycin resistant transformants selected at 30°C on LB plates with 35 µg/ml kanamycin and 0.2% (w/v) rhamnose to induce Cre expression. Kanamycin resistant transformants were streaked for single colonies onto the same selective plates and colonies were then patched on LB and LB + 64 µg/ml nourseothricin (for *nat* excision) or LB + 100 µg/ml spectinomycin (for *aadA* or *aad9* excision) plates, followed by incubation at 30°C. Nourseothricin and spectinomycin susceptible colonies indicate successful excision of the respective resistance markers. For curing of pCRE5, nourseothricin or spectinomycin susceptible colonies were streaked for single colonies on LB plates and incubated at 42°C. Cells from this culture were then streaked on LB agar plates for single colonies and the plates incubated at 42°C for two days. Single colonies growing on these plates were then patched onto LB, LB + 64 µg/ml nourseothricin (or 100 µg/ml spectinomycin) and LB + 35 µg/ml kanamycin plates. Cells growing only on the LB plates were saved as derivatives having lost the respective selection markers and were cured of pCRE5. Colony PCR was used to verify excision of the respective selection markers. Lysates were prepared by suspending a colony from a plate in 50 µl H₂O, boiling for 10 min and then centrifuging in a microfuge for 1 min at 13,000 rpm. The following primers were used: RBS Forward (5'-ACATCCGAAAGGAAACAACCATGGG) and RBS Reverse (5'-TAACCTTCGCATCTGGGCAGATGATG) for loss of *nat*; Sm^r/Spe^r-UP (5'-CGAACCCAGTGGACATAAGCC) and Sm^r/Spe^r-DN (5'-CCTGATAGTTTGGCTGTCAGC) for loss of *aadA*; and Kan-up (5'-GGGGGCCCCGGGAAAGCCAC) and Kan-dn (5'-TCCCCGGGAAGTCAGCGTAATG) for loss of the pCRE5-encoded kanamycin resistance marker.

3. Results and discussion

3.1. Construction of Nat^r, Sm^r/Spc^r and Spc^r loxP cassettes

Although the Nat, Sm/Spc and Spc resistance determinants have been employed before for genetic manipulation of bacteria, to our knowledge no versatile cassettes where they are flanked by site-specific recombinase target sites and restriction site polylinkers have been constructed. To address these points, we constructed pLNT1, pLSP1, and pLSP2 as described in the Materials and Methods section (Fig. 1). In pLNT1, *nat* gene transcription is driven from its endogenous promoter. Similarly, pLSP1 contains the *aadA* gene transcribed from its own putative promoter. The *aad9* gene contained on pLSP2 is transcribed from the *cat* gene promoter. In all three plasmids the resistance genes and their regulatory sequences

are flanked by the Cre recombinase target *loxP* and polylinker restriction sites. The latter can be used to excise the *loxP-nat-loxP*, *loxP-aadA-loxP* and *loxP-aad9-loxP* cassettes for subcloning into target DNA.

3.2. Susceptibilities of *Burkholderia* species to nourseothricin and spectinomycin

Our main research interests are studies of *B. pseudomallei* and melioidosis, the disease that it causes (Wiersinga et al., 2012). In the United States *B. pseudomallei* is a select agent whose acquisition, use, and storage requires approved BSL-3 facilities. The attenuated *B. pseudomallei* strain Bp82 was derived from virulent strain 1026b (Propst et al., 2010) and subsequently excluded from select agent regulations. It facilitates routine *B. pseudomallei* experimentation because it can be handled at BSL-2 with Institutional Biosafety Committee approval. Bp82 is a *purM* derivative of 1026b and exhibits the same antibiotic susceptibility patterns as its parental strain. *B. thailandensis* is closely related to and often used as a surrogate for *B. pseudomallei* (Brett et al., 1998; Yu et al., 2006). It also can be handled at BSL-2 and is more widely available. To assess the utility of the Nat^r and Spc^r markers in *B. pseudomallei* and *B. thailandensis*, we tested the susceptibilities of prototype and AmrAB-OprA efflux pump mutant strains to nourseothricin and spectinomycin (Table 1). It is evident that compared to the *B. thailandensis* E264 and *B. pseudomallei* 1026b prototype strains, the respective AmrAB-OprA efflux pump mutant strains (DW503, Bp50 and Bp82.27) were significantly more susceptible to these antibiotics. Spectinomycin was previously shown to be a substrate of the AmrAB-OprA efflux pump while this is the first demonstration of nourseothricin also being subjected to efflux by AmrAB-OprA. This is perhaps not too surprising since nourseothricin is a streptothricin aminoglycoside antibiotic and aminoglycosides are the preferred AmrAB-OprA efflux pump substrates (Moore et al., 1999; Trunck et al., 2009). Basal expression levels of AmrAB-OprA therefore cause intrinsic resistance to this class of compounds. Nourseothricin and spectinomycin can thus not be used as selection markers in prototype strains but in AmrAB-OprA efflux pump mutant strains. Such strains are popular because of their increased antibiotic susceptibility, which facilitates their genetic manipulation and use in cell invasion experiments (French et al., 2011; Campos et al., 2013; Podnecky et al., 2015).

3.3. Construction and use of mini-Tn7 elements with Nat^r and Sm^r/Spc^r selection markers

To assess the utilities of the Nat^r and Sm^r/Spc^r cassettes contained on pLNT1, pLSP1 and pLSP2 for engineering of genetic tools, the *loxP-nat-loxP*, *loxP-aadA-loxP* and *loxP-aad9-loxP* cassettes were used to construct the mini-Tn7 *T-nat*, and mini-Tn7 *T-aadA*, and mini-Tn7 *T-aad9* elements located on the mini-Tn7 delivery vector pUC18T (Fig. 2). The mini-Tn7 elements were first conjugally transferred to *B. thailandensis* DW503 together with the helper plasmid pTNS3 encoding the Tn7 site-specific transposition pathway and Nat^r or Spc^r transformants selected on LB plates containing either nourseothrin or spectinomycin. Selected transformants were then tested for integration at the two possible *glmS1*- or *glmS2*-associated Tn7 insertion sites (Choi et al., 2005). Fig. 3 shows the results from an experiment with mini-Tn7 *T-aadA*. In this instance, transformants that had the mini-Tn7 element either integrated at *glmS1* or *glmS2* were selected on LB plates with spectinomycin. Colony PCR was performed on boiled cell lysates from four representative transformants using primer sets $\text{P}_{\text{Sm}^r/\text{Spc}^r\text{UP}}\text{-P}_{\text{Sm}^r/\text{Spc}^r\text{DN}}$, $\text{P}_{\text{glmS1-DN}}\text{-P}_{\text{Tn7L}}$, and $\text{P}_{\text{Tn7L}}\text{-P}_{\text{glmS2-DN}}$ to test for

the presence of *aadA*, and insertion at *glmS1* or insertion at *glmS2*, respectively. The *aadA* gene was present in all *Spc^r* *B. thailandensis* DW503 transformants (Fig. 3A). In these transformants, insertions occurred primarily at the *glmS1*-associated insertion site as evidenced by the presence of a 328-bp PCR product (Fig. 3B; transformants 1, 3 and 4). As indicated by the presence of a 394-bp PCR product, only one insertion was observed at the *glmS2*-associated insertion site (transformant 2). Experiments with mini-Tn 7T-*aad9* and mini-Tn 7T-*nat* yielded similar results. With mini-Tn 7T-*aad9* five out of 12 transformants tested had mini-Tn 7T-*aad9* inserted at *glmS1* and the remaining seven transformants had double insertions at *glmS1* and *glmS2*. In the case of mini-Tn 7T-*nat* three out of five transformants had insertions at *glmS1* and the other two were at *glmS2*.

Next, the mini-Tn 7T-*nat* and mini-Tn 7T-*aadA* elements were transposed into the genome of *B. pseudomallei* (*amrRAB-oprA*) strain Bp50 and transformants were either selected on LB plates with nourseothricin or spectinomycin. The presence of the mini-Tn 7T elements at the *glmS1*-, *glmS2*- and *glmS3*-associated insertion sites was then verified by colony PCR using previously defined primer sets and conditions (Choi et al., 2008). The respective mini-Tn 7 elements were present in all *Nat^r* or *Spc^r* transformants (data not shown). Nine out of ten *Nat^r* transformants tested had mini-Tn 7T-*nat* inserted at *glmS2* and one of ten had insertions at both *glmS2* and *glmS3*. Similarly, nine of ten *Spc^r* transformants tested contained mini-Tn 7T-*aadA* insertions at *glmS2* and one transformant had an insertion at *glmS3*. The mini-Tn 7T-*aad9* elements was also transposed into Bp82 (*amrAB-oprA*) strain Bp82.27 and transformants were selected on LB plates with spectinomycin. Seven of 10 *Spc^r* transformants tested contained mini-Tn 7T-*aadA* insertions at *glmS2*, two contained insertions at *glmS3* and one transformant contained insertions at *glmS2* and *glmS3* (data not shown). These observations are consistent with previous observations that the predominant Tn 7 insertion site in *B. pseudomallei* is located immediately downstream of *glmS2* (Choi et al., 2008; Bruckbauer et al., 2015).

In summary, the experiments showed that the nourseothricin and spectinomycin resistance markers are useful for selection of the insertion of genetic elements in single copy into the genomes of *B. thailandensis* and *B. pseudomallei* AmrAB-OprA efflux pump mutants. The selection of *Nat^r* and *Spc^r* transformants is clean with no background at relatively modest antibiotic concentrations, i.e. 50 µg/ml nourseothricin and 100 µg/ml spectinomycin.

3.4. Cre recombinase-mediated marker excision from chromosomally-inserted DNA

We previously demonstrated use of Flp and Cre recombinases for derivation of markerless mutants in *B. pseudomallei* (Hoang et al., 1998; Choi et al., 2008). To assess the efficiency of Cre recombinase-mediated *Nat^r* and *Spc^r* marker excision from *B. thailandensis* and *B. pseudomallei* strains, we transformed the Cre expression plasmid pCRE5 (Choi et al., 2008) into *Nat^r* or *Spc^r* strains containing either chromosomally integrated mini-Tn 7T-*nat*, mini-Tn 7T-*aadA* or mini-Tn 7T-*aad9* elements. Excision events were monitored and pCRE5 cured following previously described procedures (Choi et al., 2008). The *Nat^r* and *Spc^r* selection markers were readily excised from the respective genomes by Cre recombinase. PCR amplification across the *loxP* scar and sequencing of the PCR fragments revealed that 85–

100% of the Nat^s and Spc^s colonies tested contained the expected deletions (data not shown).

4. Conclusions

To extend the repertoire of antibiotic selection markers for genetic manipulation of bacteria we developed versatile cassettes containing nourseothricin, streptomycin/spectinomycin and spectinomycin resistance selection markers that are encoded by the *nat*, *aadA* and *aad9* genes, respectively. These small resistance cassettes are flanked by *loxP* sites and can be excised from the respective vectors using restriction enzymes with unique cleavage sites in flanking polylinkers. To prove the utility of these cassettes, we constructed mini-Tn 7T-*nat*, mini-Tn 7T-*aadA* and mini-Tn 7T-*aad9* vectors, and transposed them to defined insertion sites into the genomes of AmrAB-OprA efflux pump-deficient *B. thailandensis* and *B. pseudomallei* strains. Transformants were readily obtained and selection was clean using modest antibiotic concentrations. While not tested here, the results with single-copy mini-Tn 7 insertions indicate that the Nat^r, Sm^r/Spc^r and Spc^r resistance cassettes could also be used in gene replacement experiments using homologous recombination. The focus of the present study was on use of the Nat^r and Spc^r elements in single-copy cloning vectors but proof-of-concept studies with both selection markers have also demonstrated that they can be used for selection and stable maintenance of multicopy plasmids (data not shown). As discussed, in *Burkholderia* species nourseothricin and spectinomycin are effluxed from the cell and its use is therefore restricted to AmrAB-OprA efflux pump mutants. However, this is not a great impediment because efflux pump mutants have been constructed that are phenotypically indistinguishable from prototype strains, e.g. they are not impaired in *in vitro* growth and virulence (Campos et al., 2013; Podnecky et al., 2015). Intrinsic resistance to aminoglycosides due to efflux pump expression in prototype bacterial strains is unique to *Burkholderia* species. Rare aminoglycoside susceptible *B. pseudomallei* have been isolated from both melioidosis patients and the environment, and susceptibility was shown to be due to: i) lack of AmrAB-OprA pump expression either due to regulatory mutations (Trunck et al., 2009) or absence of the entire *amrAB-oprM* operon because of a large genomic deletion (Trunck et al., 2009); or ii) point mutations affecting the AmrB transporter amino acid sequence (Podin et al., 2014). Many *B. mallei* strains are naturally susceptible to aminoglycosides due to absence of AmrAB-OprA. For instance, the ATCC23344 prototype strain frequently used for studies with *B. mallei* contains a 50-kb chromosomal deletion encompassing the *amrAB-oprA* operon (Nierman et al., 2004) and other strains, e.g. NCTC10229 and NCTC10247, are likely aminoglycoside susceptible because only remnants of the *amrAB-oprM* operon are present (Winsor et al., 2008). While our studies have focused on *B. pseudomallei* and its close neighbor *B. thailandensis*, we anticipate that the newly described tools will also be useful for genetic manipulation of other *Burkholderia* species, e.g. members of the clinically significant *Burkholderia cepacia* complex (Vandamme and Dawyndt, 2011). For instance, efflux-deficient - and thus aminoglycoside susceptible - mutants of *B. cenocepacia* have been developed to facilitate genetic manipulation of this species (Dubarry et al., 2010; Hamad et al., 2010). Furthermore, other bacteria, e.g. *E. coli* and even *Pseudomonas aeruginosa* wild-type strains, are generally very susceptible to aminoglycosides. We therefore anticipate that the Nat^r, Sm^r/Spc^r and Spc^r

cassettes developed in this study will be widely applicable for construction of genetic tools for and manipulation of diverse bacteria. The genetic tools described in this study are available upon request.

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Highlights

Genetic manipulation of bacteria requires antibiotic selection markers

Novel nourseothricin, streptomycin, and spectinomycin cassettes were engineered

Resistance genes can be excised from the chromosome using Cre recombinase

Mini-Tn7 vectors allow site-specific integration in Gram-negative bacteria

Selection markers were evaluated in various *Burkholderia* species

Figure 1. Maps of nourseothricin and streptomycin/spectinomycin resistance *loxP* cassette vectors

Plasmid pLOX1 contains the pACD4K-C-*loxP* (Sigma-Aldrich)-engineered 5'-CTACTTCGTATAGCATACATTATACGAAGTTAT *loxP* and 3'-ATAACTTCGTATAGCATACATTATACGAAGTTAT *loxP* sites, which show increased recombination efficiencies. The unique *EcoRI* site is used for insertion of antibiotic resistance cassettes. Plasmids pLNT1, pLSP1 and pLSP2 were obtained by insertion of the nourseothricin adenylyl transferase gene (*nat*), streptomycin/spectinomycin adenylyltransferase gene (*aadA*) or spectinomycin adenylyltransferase gene (*aad9*), respectively, into pLOX1 (see Materials and Methods section for details). Abbreviations: *bla*, Ap resistance (β -lactamase) gene; K $\rightarrow$ H, *KpnI*-*SmaI*-*BamHI*-*XbaI*-*SaI*-*PstI*-*SphI*-*HindIII*.

Figure 2. Maps of pUC18T-mini-Tn7T and mini-Tn7T-*nat*, mini-Tn7T-*aadA* and mini-Tn7T-*aad9*
 The *nat*, *aadA* and *aad9* selection markers were cloned into the *EcoRV* site in the multiple cloning site of the mobilizable pUC18T-mini-Tn7T (A) to derive mini-Tn7 delivery vectors with the genetic makeup shown in (B). Abbreviations: *aadA* and *aad9*, adenylyltransferase-encoding gene; *loxP*, Cre recombinase recombination sites; *NsiI-StuI*, multiple cloning site containing the restriction enzyme cleavage sites *NsiI-SacI-SpeI-BamHI-SmaI-PstI-EcoRI-XhoI-HindIII-ApaI-KpnI-NruI-StuI* in the indicated order; *nat*, nourseothricin adenylyltransferase-encoding gene; T, transcriptional terminators T₀ and T₁ from bacteriophage λ and *E. coli rrnB* operon, respectively; Tn7L and Tn7R, left and right ends of transposon Tn7. Note: not all MCS restriction sites are unique in the mini-Tn7 transposons. In mini-Tn7T-*nat*, *BamHI*, *EcoRI*, *KpnI*, *PstI* and *SmaI* and in mini-Tn7T-*aadA*, *EcoRI* and *KpnI* occur elsewhere in the respective transposons. Similarly, mini-Tn7T-*aad9* contains two additional *KpnI* sites flanking the *aad9* gene.

Fig. 3. Testing of mini-Tn7T-*aadA* insertion *B. thailandensis*

Boiled cell lysates were prepared from four randomly chosen spectinomycin resistant transformants of strain DW503 and analyzed for the presence of mini-Tn7T-*aadA* by PCR using primer pair Sm^r/Spe^r-UP and Sm^r/Spe^r-DN to detect *aadA* (A) and primer pairs P_{glmS1}-DN-P_{Tn7} or P_{Tn7}-P_{glmS2}-DN for insertion at either *glmS1* or *glmS2*, respectively (B). (A) Lane C, positive control pUC18T-mini-Tn7T-*aadA*; lane 1, negative control DW503 without mini-Tn7 insertion; lanes 2–5, randomly chosen transformants #1–#4. (B) Lanes 1, 3, 5 and 7 are transformants #1–#4 analyzed with primer pair P_{glmS1}-DN-P_{Tn7}; lanes 2, 4, 6 and 8 are transformants #1–#4 analyzed with primer pair P_{Tn7}-P_{glmS2}-DN. Lanes M in both panels contained Hi-LO DNA marker from Minnesota Molecular (Minneapolis, MN).

Table 1Antibiotic susceptibilities of *B. thailandensis* and *B. pseudomallei* strains

Strain	MIC (µg/ml)	
	Nourseothricin	Spectinomycin
<i>B. thailandensis</i>		
E264	>512	256
DW503 [E264 (<i>amrRAB-oprA</i>)]	8	4
<i>B. pseudomallei</i>		
1026b	>1,024	2,048
Bp50 [1026b (<i>amrRAB-oprA</i>)]	8	64
Bp82	512	1024
Bp82.27 [Bp82 (<i>amrAB-oprA</i>)]	4	32

Minimal inhibitory concentrations (MIC) were determined using the broth microdilution method.