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 resistant 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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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 pHP45Ω (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 adenyltransferase encoded by the

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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 DH5α (Liss, 1987) and RHO3 (Lopez et al., 2009). *Burkholderia* strains used were *B. thailandensis* E264 (Brett et al., 1998) and its Δ(*amrRAB-oprA*) efflux pump mutant derivative DW503 (Burtnick et al., 2001), and *B. pseudomallei* strains 1026b (DeShazer et al., 1997) and Δ(*amrRAB-oprA*) efflux pump mutant derivative Bp50 (Choi et al., 2008), and Bp82 (Propst et al., 2010) and its Δ(*amrRAB-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 Δ(*amrRAB-oprA*) derivative Bp82.27 were grown in media supplemented with 80 μg/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 Δ*asd* mutant and requires 400 μg/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 μg/ml ampicillin, 32 μg/ml nourseothricin and 100 μg/ml spectinomycin for *E. coli*; 32 μg/ml nourseothricin and 100 μg/ml spectinomycin for *B. thailandensis* and *B. pseudomallei* Δ(*amrRAB-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-Tn7T (Choi and Schweizer, 2006). The GenBank accession numbers for these plasmids are EU215436 for pLOX1; EU215432 for pTNS3; and AY599230 for pUC18T-mini-Tn7T.

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 1109-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 1129-bp *Eco*RI fragment which was gel-purified and then ligated into the *Eco*RI 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 1163-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 1183-bp *Eco*RI 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 *Eco*RI 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 *Eco*RI fragment, which was gel-purified and then ligated into the *Eco*RI 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 1322-bp *Xba*I fragment blunt-ended with T4 DNA polymerase and containing *loxP-nat-loxP* from pLNT1 was subcloned into the *Eco*RV 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 1350-bp *Sma*I fragment containing *loxP-aadA-loxP* from pLSP1 or a 1081-bp *Sma*I fragment containing *loxP-aad9-loxP* from pLSP2 into the *Eco*RV 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-Tn7-*aadA* or pUC18T-mini-Tn7-*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, streptomycin/spectinomycin, and spectinomycin resistance markers