Method for Regulated Expression of Single-Copy Efflux Pump Genes in a Surrogate *Pseudomonas aeruginosa* Strain: Identification of the BpeEF-OprC Chloramphenicol and Trimethoprim Efflux Pump of *Burkholderia pseudomallei* 1026b

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Construction and integration of recombinant mini-Tn7 expression vectors into the chromosome of a surrogate, efflux-sensitized, and biosafe *Pseudomonas aeruginosa* host was validated as a generally applicable method for studies of uncharacterized bacterial efflux pumps. Using this method, the *Burkholderia pseudomallei* bpeEF-oprC operon was shown to encode a chloramphenicol and trimethoprim efflux pump.

Multidrug resistance pumps play major roles in intrinsic and acquired bacterial antibiotic resistance and also in bacterial pathogenicity (13). A major handicap associated with the characterization of bacterial efflux pumps is that they are under very tight regulatory control and thus considered “silent” in wild-type strains because inducing conditions are usually unknown. For this reason, such endeavors are restricted to clinical or laboratory-induced mutants overexpressing these pumps, but such mutants are scarce in many bacterial species, especially those whose use is restricted or those that are difficult to cultivate and genetically modify. In this study, we describe a method that may have widespread use in the study of uncharacterized bacterial efflux pumps. The method employs a novel mini-Tn7-based gene integration system developed in our laboratory (3) and a surrogate, drug-susceptible *Pseudomonas aeruginosa* strain which allows regulated gene expression from an unmarked, single-copy, chromosomally integrated recombinant construct. Here, we test the method by cloning, expressing, and functionally characterizing a new resistance nodulation cell division (RND) chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. In strain K96243, this pump is encoded by the BPSS0292-BPSS0293-BPSS0294 genes, and in 1710b, a strain more closely related to 1026b than K96243, the same pump is encoded by the genes annotated as ceoA-ceoB-BURPS1710b_A1842 (Fig. 1). In both strains, these genes are located on chromosome II, albeit in two different regions of the chromosome. These RND efflux pump genes are parts of operons which also contain genes encoding lipase-like proteins, BPSS0291 in K96243 and llpE in 1710b (Fig. 1). Upstream of these operons, and transcribed divergently from them, are BPSS0290 and ceoR, respectively, which encode LysR type regulatory proteins. The transcriptional organization of this region of *B. pseudomallei* K29243 chromosome II is reminiscent of the *Burkholderia cenocepacia* ceoA-BopcM efflux pump genes, which are part of a transcriptional unit with the upstream, lipase-like-protein-encoding llpE gene (11). Expression of the llpE-ceoA-BopcM operon is believed to be under the transcriptional control of a LysR type regulator encoded by the upstream and divergently transcribed ceoR gene. It is therefore likely that the BPSS0292-BPSS0293-BPSS0294 genes and ceoA-ceoB-BURPS1710b_A1842 encode a drug efflux pump, which will hereafter be named BpeEF-OprC for all *B. pseudomallei* strains to comply with established *B. pseudomallei* efflux pump nomenclature.

The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were routinely grown in Luria-Bertani (LB) medium (EM Sciences, Gibbstown, NJ). Growth medium was supplemented with ampicillin (Sigma, St. Louis, MO) (100 µg/ml) for the selection of *Escherichia coli* strains containing plasmids carrying the ampicillin resistance marker. Fosmid-containing *E. coli* strains were grown in LB broth supplemented with 12 µg/ml of chloramphenicol (Sigma). Induction of fosmids to attain multiple copies was performed by adding 0.2% L-arabinose (Eastman Chemicals, Rochester, NY) to the growth medium. Gentamicin (Gm)-resistant *P. aeruginosa* strains were selected on LB plates containing 15 µg/ml Gm (Sigma) (LB+Gm15 plates).

Fosmid clones of a *B. pseudomallei* 1026b library containing contigs corresponding to the location of bpeEF-oprC on *B. pseudomallei* K96243 were obtained from the University of Washington Genome Sequencing Center and used for PCR amplification of portions of the bpeEF-oprC operon. PCR primers were designed based on the *B. pseudomallei* K96243 sequence available from GenBank. The following primers were used to introduce the restriction sites, indicated by underlined bases and denoted in parentheses (base changes introduced to generate new restriction sites are lowercase): BpeEFC (CAT CCGAATTCTAGAACAACCGC) (EcoRI), BpeEFCR (GCCG CCGacAGTTCAACGCG) (HindIII), BpeBgF (CGACACGAGATACATACC) (BglII), and BpeBgR (GGTAGATCTGATCCTGTTG) (BglII). Under standard PCR conditions for G+C-rich DNA (7), primer sets BpeEFC-E and BpeBg-F and BpeEFCR and BpeBg-R were used to amplify two fragments of

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inducible by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (Gold Biotechnology, St. Louis, MO) to the growth medium. The integrity of the entire bpeEF-oprC operon was determined by sequencing at the Colorado State University Macromolecular Resources core facility. Sequence alignments showed that the B. pseudomallei 1026b bpeEF-oprC sequence is nearly identical (with a difference in 19 out of 6,020 nucleotides within the sequenced DNA) to the bpeEF-oprC operon of strain K96243. Open reading frame predictions showed that only 3 of the 9 base changes resulted in amino acid changes.

A surrogate, drug-susceptible, and biosafe P. aeruginosa strain was constructed by transforming PAO397 with a suicide plasmid containing the previously described unmarked ΔpscC allele (15) with a rapid electroporation method (4). Chromosomal plasmid integration events were selected by plating the transformation mixture on LB + Gm15 plates, and the resulting merodiploids were resolved by streaking transformants on LB plates supplemented with 5% sucrose. Gm-susceptible colonies were then analyzed for the deletion of the pscC gene by colony PCR (7) with primers PscB-F (ATGGATCATCTGTTACGACGACGGG) and PscC-R (ACCAGGCCTCTTTGAGGGA). The PCR fragment of expected size from one of the colonies was sequenced to confirm the deletion of 1,673 bp from the pscC gene. One strain containing the correct ΔpscC allele was retained and named PAO750. This strain is highly drug susceptible by virtue of deletions of five operons encoding RND pumps as well as the opmH gene encoding the P. aeruginosa TolC homolog that was shown to function with various RND pumps (6, 8). Deletion of the pscC gene, which encodes the outer membrane component of the sole P. aeruginosa type III secretion system, renders the organism avirulent (15) and makes it thus a suitable biosafe host strain for the cloning of DNA segments from a category B pathogen.

Insertion of bpeEF-oprC into the PAO750 genome was performed using the mini-Tn7 system as previously described (3, 5) and is illustrated in Fig. 2. Briefly, competent PAO750 cells were electroporated with 50 ng each of pPS1738 and the helper plasmid pTNS2. Transformants were selected on LB + Gm15 plates, and the Gm marker was subsequently deleted using Flp transposition of expected size from one of the colonies was sequenced to confirm the deletion of 1,673 bp from the pscC gene. One strain containing the correct ΔpscC allele was retained and named PAO750. This strain is highly drug susceptible by virtue of deletions of five operons encoding RND pumps as well as the opmH gene encoding the P. aeruginosa TolC homolog that was shown to function with various RND pumps (6, 8). Deletion of the pscC gene, which encodes the outer membrane component of the sole P. aeruginosa type III secretion system, renders the organism avirulent (15) and makes it thus a suitable biosafe host strain for the cloning of DNA segments from a category B pathogen.

Insertion of bpeEF-oprC into the PAO750 genome was performed using the mini-Tn7 system as previously described (3, 5) and is illustrated in Fig. 2. Briefly, competent PAO750 cells were electroporated with 50 ng each of pPS1738 and the helper plasmid pTNS2. Transformants were selected on LB + Gm15 plates, and the Gm marker was subsequently deleted using Flp

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recombinase (5), yielding PAO789. The drug susceptibility pattern of PAO789 was assessed by determining MICs on Mueller-Hinton broth (Difco, Becton-Dickinson, Sparks, MD)-grown cells with the twofold broth microdilution technique, following National Committee for Clinical Laboratory Standards (NCCLS) guidelines (12) or by the Etest method (AB Biodisk, Piscataway, NJ) (ciprofloxacin only). Induction of BpeEF-OprC expression in PAO789 with 1 mM IPTG resulted in a significant (fourfold) increase in the MICs for chloramphenicol and trimethoprim, but no change was observed in MICs for the other antibiotics and antimicrobials tested (Table 2). Addition of the known RND efflux pump inhibitor Phe-Arg-
/naphthylamide dichloride (Sigma) (9) to IPTG-induced cells at a final concentration of 10
/g/ml caused an 8- to 16-fold decrease in the MICs for chloramphenicol and trimethoprim. These data indicate that BpeEF-OprC is a chloramphenicol and trimethoprim efflux pump of possible clinical significance because both of these antibiotics have been used, for eradication and acute-phase melioidosis therapies, respectively (2).

The method described in this study may have widespread use in the characterization of efflux pumps from bacterial pathogens. To avoid interference between resistance determinants contained on the mini-Tn7 elements and those provided by the RND pumps, several mini-Tn7-LAC expression vectors with diverse selection markers were constructed (data not shown). Single-copy insertion and inducible efflux pump expression are desirable features since the presence of RND efflux operons on multicopy plasmids is often not well tolerated by bacteria. Expression from a regulated promoter also circumvents the reliance on clinical or laboratory-induced-pump-overexpressing mutants for characterization of the substrate profiles and possible clinical significance of uncharacterized pumps. This method may also be used for rapid characterization of clinical isolates that exhibit multidrug resistance phenotypes, by

**FIG. 2.** Single-copy integration of the *B. pseudomallei* bpeEF-oprC operon into the genome of a surrogate *P. aeruginosa* strain. The mini-Tn7 suicide delivery plasmid (double line) harboring the recombinant mini-Tn7 element (bold line) flanked by the left and right Tn7 ends (Tn7L and Tn7R) and a helper plasmid encoding the site-specific Tn7 transposition pathway (+TnsABCD) were coelectroporated into PAO750, and gentamicin-resistant (Gm') transformants were selected. One such transformant (PAO783) had the mini-Tn7 integrated into the PAO750 chromosome (stippled line) downstream of the glmS gene. The Gm' determinant encoded by the aacC1 gene flanked by Flp recombinase targets (FRT) was subsequently deleted from the PAO783 chromosome by using Flp recombinase, which resulted in an unmarked strain (PAO789) in which bpeEF-oprC expression is under the control of the tac promoter (P_tac), whose activity is regulated by the Lac repressor encoded by lacIq. Other abbreviations: bla, β-lactamase-encoding gene; ori, ColE1-derived origin of replication.

**TABLE 2.** MICs of different antibiotics for PAO789<sup>a</sup>

| Strain                  | MIC (µg/ml) of: |
|-------------------------|-----------------|
|                          | Ch  | Tp  | Gm  | Km  | Str | Tri | EtBr | Cip  |
| PAO789                  | 2   | 2   | 2   | 128 | 8   | 4   | 64   | 0.06 |
| PAO789 (+1 mM IPTG)     | 8   | 8   | 2   | 128 | 8   | 4   | 64   | 0.06 |
| PAO789 (+1 mM IPTG + 10 µg/ml PAN) | 0.5 | 1   | ND  | ND  | ND  | ND  | ND   | ND   |

<sup>a</sup> All MIC determinations were performed using the broth microdilution technique or the Etest method (ciprofloxacin only) and Mueller-Hinton broth as the growth medium. Abbreviations: Ch, chloramphenicol; Tp, trimethoprim; Km, kanamycin; Str, streptomycin; Tri, triclosan; EtBr, ethidium bromide; Cip, ciprofloxacin; PAN, Phe-Arg-β-naphthylamide dichloride; ND, not determined.
matching the antibiotic resistance profile of the clinical isolates with those established for different pumps of that organism. By using a panel of strains expressing different pumps, one pump at a time, the method may also be applicable to the screening and identification of broad-spectrum efflux pump inhibitors. One possible drawback of using the testing and screening approach with a surrogate *P. aeruginosa* strain may be the inability to correctly identify peptide substrates and inhibitors that actually work in strains which are naturally resistant to peptide antibiotics, e.g., polymyxin-resistant *Burkholderia* spp. Lastly, the data obtained in this study further increase our understanding of the role that RND pumps play in clinically significant intrinsic and acquired antibiotic resistance, a step towards development of sorely needed improved therapies for melioidosis. In addition to AmrAB-OprA (10) and BpeAB-OprB (1), which export aminoglycosides and macrolides, BpeEF-OprC is the third example of a *B. pseudomallei* RND pump exporting clinically significant antibiotics.

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REFERENCES

1. Chan, Y. Y., T. M. C. Tan, Y. M. Ong, and K. L. Chua. 2004. BpeAB-OprB: a multidrug efflux pump in *Burkholderia pseudomallei*. Antimicrob. Agents Chemother. 48:1128–1135.

2. Cheng, A. C., and B. J. Currie. 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. 18:383–416.

3. Choi, K.-H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. Karkhoff-Schweizer, and H. P. Schweizer. 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat. Methods 2:443–448.

4. Choi, K.-H., A. Kumar, and H. P. Schweizer. 2006. A 10 min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosones and plasmid transformation. J. Microbiol. Methods 64:391–397.

5. Choi, K.-H., and H. P. Schweizer. 2006. mini-Tn7 insertion in bacteria with single attB sites: example *Pseudomonas aeruginosa*. Nat. Protocols 1:153–161.

6. Chuanchuen, R., T. Murata, N. Gotoh, and H. P. Schweizer. 2005. Substrate-dependent utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexUK efflux pump. Antimicrob. Agents Chemother. 49:2133–2136.

7. Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212:77–86.

8. Jo, J. T. H., F. S. Brinkman, and R. E. W. Hancock. 2003. Aminoglycoside efflux in *Pseudomonas aeruginosa*: involvement of novel outer membrane proteins. Antimicrob. Agents Chemother. 47:1101–1111.

9. Lomovskaya, O., M. S. Warren, A. Lee, J. Galazzo, R. Franko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. 45:105–116.

10. 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.

11. Nair, B. M., K. J. Cheung, Jr., A. Grifith, and J. L. Burns. 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genome var III (B. cenocepacia). J. Clin. Investig. 113:846–473.

12. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6 and MIC testing supplemental tables M100-S13, 6th ed., vol. 23, no. 2. National Committee for Clinical laboratory Standards, Wayne, Pa.

13. Nair, B. M., K. J. Cheung, Jr., A. Grifith, and J. L. Burns. 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genome var III (B. cenocepacia). J. Clin. Investig. 113:846–473.

14. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A6 and MIC testing supplemental tables M100-S13, 6th ed., vol. 23, no. 2. National Committee for Clinical laboratory Standards, Wayne, Pa.

15. Piddock, L. J. 2006. Multidrug-resistance efflux pumps—not just for resistance. Nat. Rev. Microbiol. 4:629–636.

16. West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene 128:81–86.

17. Wolfgang, M. C., V. T. Lee, M. E. Gilmore, and S. Lory. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependant signaling pathway. Dev. Cell 4:253–263.