ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2006, p. 3460–3463 Vol. 50, No. 10
0066-4804/06/$08.00+0 doi:10.1128/AAC.00440-06

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

Ayush Kumar,[1] Kim-Lee Chua,[2] and Herbert P. Schweizer[1]*

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado 80523-1682,[1] and Department of Biochemistry, National University of Singapore, Singapore[2]

Received 7 April 2006/Returned for modification 18 June 2006/Accepted 21 July 2006

Construction and integration of recombinant mini-Tn*7* 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-Tn*7*-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 ceoAB-opcM* efflux pump genes, which are part of a transcriptional unit with the upstream, lipase-like-protein-encoding *llpE* gene (11). Expression of the *llpE-ceoAB-opcM* 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 for *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): BpeEFEc (CAT CCGAATTCAGAACAACCG) (EcoRI), BpeEFCR (GCCG CCGaAgcTTCAACGCG) (HindIII), BpeBgF (CGACACGA TGCAGATCTACC) (BglII), and BpeBgR (GGTAGATCTG CATCGTGTCG) (BglII). Under standard PCR conditions for G+C-rich DNA (7), primer sets BpeEFEc and BpeBgR and BpeEFCR and BpeBgF were used to amplify two fragments of

* Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682. Phone: (970) 491-3536. Fax: (970) 491-1815. E-mail: Herbert.Schweizer@colostate.edu.

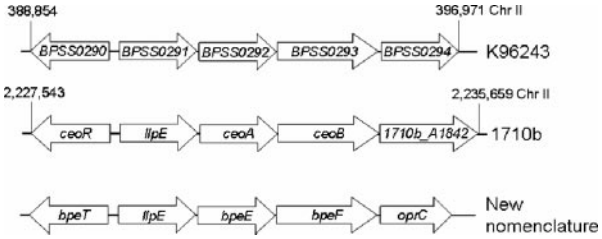

FIG. 1. Organization of the regions containing the *bpeEF-oprC* genes in different *B. pseudomallei* strains. In both strains analyzed, the *bpeEF-oprC* genes are located on chromosome II (Chr II), albeit in two different regions of the chromosomes. The indicated coordinates and gene annotations were taken from published sequences for strains K96243 (GenBank accession number NC 006351) and 1710b (GenBank accession number NC 007435). To comply with established *B. pseudomallei* efflux pump nomenclature, the BPSS0292-BPSS0293-BPSS0294 and *ceoA-ceoB*-BURPS1710b_A1842 genes were renamed *bpeE-bpeF-oprC*. Similarly, the proposed LysR type BPSS0290 and *ceoR* regulatory genes were renamed *bpeT*. BPSS0291 and *llpE* encode a protein with high similarity to lipase-like protein E from *B. cenocepacia* and were therefore named *llpE*.

3,632 bp and 2,479 bp, respectively. The 3,632-bp fragment contained the membrane fusion protein-encoding gene, *bpeE*, and a portion of the RND pump-encoding gene, *bpeF*, while the 2,479-bp fragment contained the remainder of the *bpeF* gene and the outer membrane component-encoding gene, *oprC*. Both fragments were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) by utilizing *E. coli* Top10F′ as the host, following the manufacturer's instructions. The *bpeEF-oprC* operon was then assembled in its entirety in the cloning vector pUCP20 (14) to yield pPS1679 (bacterial strains and plasmids are listed in Table 1). The complete *bpeEF-oprC* operon was isolated from pPS1679 by digestion with EcoRI (blunt ended with T4 polymerase [NEB, Beverly, MA]) and HindIII, and the resulting fragment was subsequently ligated into the SmaI/HindIII-digested pUC18-mini-Tn*7*T-LAC vector to yield pPS1738 (Fig. 2). In this plasmid, expression of the *bpeEF-oprC* operon is driven from the *tac* promoter, which is controlled by the *lacI*^{q}-encoded Lac repressor and therefore 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 Macromoleular 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 19 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 Δ*pscC* 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 (ATGGATCATCTGTT GAGCGG) and PscC-R (ACCAGGCGCCGTCTTTGGGA). 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 Δ*pscC* 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-Tn*7* 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

TABLE 1. Bacterial strains and plasmids used in this study

Plasmid or strain	Relevant characteristics[a]	Source or reference
Plasmids		
pCR2.1	Ap^r; PCR cloning vector	Invitrogen
pUCP20	Ap^r; *E. coli-P. aeruginosa* shuttle vector	14
pPS1679	Ap^r; pUCP20 containing the *bpeEF-oprC* operon of *B. pseudomallei* 1026b	This study
pUC18-mini-Tn*7*T-LAC	Ap^r Gm^r; mini-Tn*7* expression vector containing *lacI*^{q} and *tac* promoter	3
pPS1738	Ap^r Gm^r; pUC18-mini-Tn*7*-LAC containing the *bpeEF-oprC* operon of *B. pseudomallei* 1026b	This study
pEXGm-Δ*pscC*	Ap^r; gene replacement vector containing unmarked *P. aeruginosa* Δ*pscC* allele	15
pTNS2	Ap^r; helper plasmid encoding the site-specific TnsABCD Tn*7* transposition pathway	3
pFLP2	Ap^r; source of Flp recombinase	7
P. aeruginosa strains		
PAO397	Δ(*mexAB-oprM*) Δ(*mexAB-oprM*) Δ*(mexCD-oprJ)* Δ(*mexEF*-oprN) Δ(*mexJK*) Δ(*mexXY*) Δ*opmH*	6
PAO750	PAO397 with Δ*pscC*	This study
PAO783	Gm^r; PAO750 with chromosomally integrated mini-Tn*7*T-LAC-*bpeEF-oprC*	This study
PAO789	PAO783 without the Gm^r marker	This study

[a] Abbreviations: Ap, ampicillin; Gm, gentamicin; r, resistance.

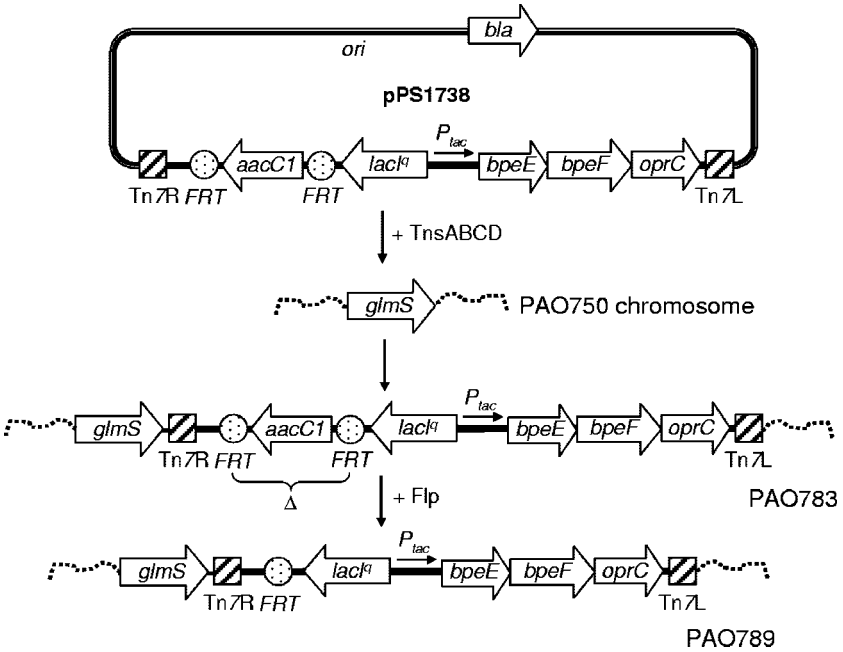

FIG. 2. Single-copy integration of the *B. pseudomallei bpeEF-oprC* operon into the genome of a surrogate *P. aeruginosa* strain. The mini-Tn*7* suicide delivery plasmid (double line) harboring the recombinant mini-Tn*7* element (bold line) flanked by the left and right Tn*7* ends (Tn*7*L and Tn*7*R) and a helper plasmid encoding the site-specific Tn*7* transposition pathway (+TnsABCD) were coelectroporated into PAO750, and gentamicin-resistant (Gm^r) transformants were selected. One such transformant (PAO783) had the mini-Tn*7* integrated into the PAO750 chromosome (stippled line) downstream of the *glmS* gene. The Gm^r 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 $lacI^q$. Other abbreviations: *bla*, β-lactamase-encoding gene; *ori*, ColE1-derived origin of replication.

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-β-naphthylamide dichloride (Sigma) (9) to IPTG-induced cells at a final concentration of 10 μg/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-Tn*7* elements and those provided by the RND pumps, several mini-Tn*7*-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

TABLE 2. MICs of different antibiotics for PAO789[a]

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

[a] 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 *B. pseudomallei* 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.

This work was supported by NIH grant AI065357.

We thank M. Jacobs from the University of Washington Genome Sequencing Center for providing fosmids containing *B. pseudomallei* genomic DNA.

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