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BpeAB-OprB, a Multidrug Efflux Pump in *Burkholderia pseudomallei*

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is intrinsically resistant to a wide range of antimicrobial agents, including β-lactams, aminoglycosides, macrolides, and polymyxins. An operon, *bpeR-bpeA-bpeB-oprB*, which encodes a putative repressor, a membrane fusion protein, an inner membrane protein, and an outer membrane protein, respectively, of a multidrug efflux pump of the resistance-nodulation-division family was identified in *B. pseudomallei*. The divergently transcribed *bpeR* gene encodes a putative repressor protein of the TetR family which probably regulates the expression of the *bpeAB-oprB* gene cluster. Comparison of the MICs and minimal bactericidal concentrations of antimicrobials for *bpeAB* deletion mutant KHWΔ*bpeAB* and its isogenic wild-type parent, KHW, showed that the *B. pseudomallei* BpeAB-OprB pump is responsible for the efflux of the aminoglycosides gentamicin and streptomycin, the macrolide erythromycin, and the dye acriflavine. Antibiotic efflux by the BpeAB-OprB pump was dependent on a proton gradient and differs from that by the AmrAB-OprA pump in that it did not efflux the aminoglycoside spectinomycin or the macrolide clarithromycin. The broad-spectrum efflux pump inhibitor MC-207,110 did not potentiate the effectiveness of the antimicrobials erythromycin and streptomycin in *B. pseudomallei*.**

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative soil bacillus endemic mainly in Southeast Asia and northern Australia; but cases have also been reported in India, China, Taiwan, and Laos (6). The disease may manifest itself as an acute, subacute, or chronic form; and the acute form of melioidosis is often fatal, despite aggressive antibiotic treatment. Treatment of severe melioidosis includes a combination of cefoperazone-sulbactam plus co-trimoxazole or ceftazidime plus co-trimoxazole (4). A high-dose intravenous ceftazidime regimen was shown to be superior to the conventional four-drug regimen (chloramphenicol, doxycycline, and trimethoprim-sulfamethoxazole) (3). Despite treatment with high-dose ceftazidime, severe melioidosis carries a mortality rate of 40% (2).

Antibiotic-resistant *B. pseudomallei* strains are known to emerge during the treatment of melioidosis. Such chloramphenicol- and ceftazidime-resistant *B. pseudomallei* strains were found to be fully virulent and frequently showed cross-resistance to other antimicrobials such as tetracyclines, sulfamethoxazole, trimethoprim, and ciprofloxacin (7).

The low success rate of the treatment of melioidosis is attributed to the fact that *B. pseudomallei* is intrinsically resistant to a variety of antibiotics, including β-lactams, aminoglycosides, macrolides, and polymyxins (10). Broadly specific efflux systems which are able to accommodate a variety of unrelated antimicrobial agents, including antibiotics, biocides, dyes, detergents, fatty acids, organic solvents, and homoserine lactones, are responsible for much of the intrinsic multidrug resistance in gram-negative bacteria (22). In *B. pseudomallei*, AmrAB-OprA, an efflux system of the resistance-nodulation-division (RND) family, has been reported to be responsible for the efflux of aminoglycosides and macrolides (18). Other members of the RND family which are responsible for the efflux of antimicrobials in gram-negative bacteria include AcrAB-TolC of *Escherichia coli*; the AcrAB homologue of *Salmonella enterica* serovar Typhimurium; MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM of *Pseudomonas aeruginosa*; and CeoAB-OpcM of *Burkholderia cepacia* (22).

In this study, we describe the identification in *B. pseudomallei* of the gene operon *bpeR-bpeA-bpeB-oprB*, which encodes a multidrug efflux system of the RND family. Gene localization, substrate specificities, and proton gradient dependence distinguish the *B. pseudomallei* BpeAB-OprB efflux system from that of AmrAB-OprA, although both systems efflux aminoglycosides and macrolides.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, cultures were grown under aerobic conditions at 37°C in Luria-Bertani (LB) agar or LB broth (Becton Dickinson, Cockeysville, Md.). The antibiotic concentrations used for *E. coli*, when it was used, were as follows: ampicillin, 50 μg/ml; gentamicin, 50 μg/ml; trimethoprim, 25 μg/ml; kanamycin, 10 μg/ml; streptomycin, 50 μg/ml. Those used for *B. pseudomallei* were as follows: kanamycin, 200 μg/ml; trimethoprim, 100 μg/ml.

Construction and screening of the *B. pseudomallei* DNA library. A genomic library of *B. pseudomallei* ATCC 23343 (American Type Culture Collection [ATCC], Manassas, Va.) was constructed by using partially digested Sau3AI genomic DNA and XhoI-digested arms of bacteriophage λGem-12 (Promega, Madison, Wis.). DNA manipulation techniques, plaque hybridization, and extraction of bacteriophage DNA were performed as described by Sambrook and Russell (27), while the extraction of bacterial genomic DNA was done by the method described by Pitcher et al. (21). Two genomic clones were identified from a screen by using ^{32}P-radiolabeled *B. cepacia ceoA*- and *ceoB*-specific DNA probes (Amersham Biosciences, Little Chalfont, United Kingdom), which were generated with the primers pairs ceoA3F-ceoA4R and ceoB6F-ceoB4R, respectively (Table 2).

The *bpeR-bpeAB-oprB* contig was assembled from sequences of the *ceoB* clone obtained by a combination of subcloning into pBluescriptII (Stratagene, La Jolla, Calif.) and primer walking (Fig. 1). Plasmid DNA was purified from overnight cultures with a Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany). PCR was performed in a PTC-100 Peltier thermal cycler (MJ Research, Waltham, Mass.) in Mg^{2+}-free buffer containing 100 ng of template DNA, 200 μmol (each)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli		
DH5αλpir	DH5α with a λ prophage carrying the gene encoding the p protein; Kan^s Tmp^s Gen^s	16
LE392	Permissive host strain for genomic library	Promega
B. pseudomallei		
ATCC 23343	ATCC strain for genomic library generation	ATCC
KHW	Wild-type clinical isolate; Kan^s Tmp^s Gen^r	5
KHWΔ*bpeAB*	KHW with Δ*bpeAB*; Kan^r Tmp^s Gen^r	This study
KHWΔ*bpeAB*(pUCP28T*bpeAB*)	KHWΔ*bpeAB* complemented in *trans* with pUCP28T*bpeAB*; Kan^r Tmp^r Gen^r	This study
Plasmids		
pGEM-T	Vector for PCR cloning; Amp^r	Promega
pJQ200mp18	Mobilizable allelic exchange vector; *traJ sacB* Gen^r	25
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600; *ori* Tmp^r	34
pJQ200Δ*bpeAB*Km	pJQ200mp18 derivative carrying a 3.5-kb ApaI-SpeI fragment containing 5′ *bpeA*-Km^r cassette-3′ *bpeB* from pGEMTΔ*bpeAB*Km inserted into the SmaI site	This study
pUCP28T*bpeAB*	4.9-kb *bpeAB* PCR product cloned via blunt-end ligation into pUCP28T; Tmp^r	This study
pUTKm	Source of kanamycin resistance cassette; *oriR6K mobRP4* Kan^r Amp^r	8

[a] Kan, kanamycin; Tmp, trimethoprim; Gen, gentamicin; Amp, ampicillin; r, resistant; s, sensitive.

deoxynucleoside triphosphate, 50 pmol of each primer, 1.5 mM $MgSO_4$, and 0.5 U of *Tth* polymerase (Biotools, Madrid, Spain) in a total volume of 50 μl. The cycling parameters included 1 cycle at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s (with 30 s of annealing at the respective temperatures; 1 min/kb of product length at 72°C) and a final extension at 72°C for 10 min. The annealing temperatures and the extension times for the different primer pairs are listed in Table 2.

DNA sequencing was performed with a ABI BigDye (version 3) dye terminator kit, and the DNA sequence was analyzed with an automatic sequencer (model 377; Applied Biosystems, Foster City, Calif.) (29). Multiple-sequence alignments of the translated amino acid sequences of *B. pseudomallei* ATCC 23343 *bpeR-bpeAB-oprB* with RND efflux pump components from other gram-negative bacteria were performed with the AlignX program in Vector NTI Suite 7 software (Informax Inc., Bethesda, Md.) and the BLASTX program (1). Prediction of the transmembrane domain was performed with TMHMM (version 2.0) software (17).

Construction of KHWΔ*bpeAB*. The *bpeAB* deletion was generated in *B. pseudomallei* KHW, a virulent clinical isolate, by the homologous gene replacement strategy described previously (5). Briefly, *bpeA* (1.2 kbp) and *bpeB* (1.3 kbp) PCR products, obtained with primer pairs AcrAHisF-AcrAHisR and AcrB3′3716-AcrB3′R1, respectively, were digested with ClaI, which cleaved the *bpeA* and *bpeB* fragments internally. A 271-bp *bpeA* 5′ fragment and a 989-bp *bpeB* 3′ fragment were recovered after electrophoresis in 1% agarose with the GeneClean II kit (Bio 101, Inc., Vista, Calif.) and ligated with T-tailed plasmid pGEM-T (Promega) to yield pGEMTΔ*bpeAB*. Next, ClaI-linearized pGEMTΔ*bpeAB* was made blunt ended before ligation with an end-filled 2.3-kbp EcoRI fragment containing the kanamycin resistance gene cassette from pUTKm (GenBank accession number AF102233), yielding pGEMTΔ*bpeAB*Km. Conversion of DNA fragments with 3′ recessed or overhanging ends to blunt ends with T4 DNA polymerase was performed as described by the manufacturer (Promega).

A 3.5-kbp *Apa*I-*Spe*I fragment containing the 5′ *bpeA*, 3′ *bpeB*, and kanamycin resistance cassette from pGEMTΔ*bpeAB*Km was made blunt ended and inserted into SmaI-linearized pJQ200mp18, yielding pJQ200Δ*bpeAB*Km. pJQ200Δ*bpeAB*Km was first introduced into *E. coli* DH5λpir by electroporation with a MicroPulser instrument (Bio-Rad, Hercules, Calif.) and then mobilized into *B. pseudomallei* KHW by triparental mating with *E. coli* HB101(pRK600) as the helper strain, as described by de Lorenzo et al. (8). Exconjugants were plated on LB agar containing kanamycin, streptomycin, and 5% (wt/vol) sucrose to select for recombinants which had undergone allelic exchange. The chromosomal deletion of *bpeAB* was confirmed by PCR and Northern blotting. PCR amplification with primers AcrABpro and AcrB3′R1 (Table 2; Fig. 1) yielded a 4.9-kbp fragment from KHW and a 3.5-kbp fragment from KHWΔ*bpeAB*, consistent with a 3.7-kbp chromosomal deletion of *bpeA-bpeB* and replacement with a 2.3-kbp kanamycin resistance cassette (data not shown). The insertion of the kanamycin cassette in the *bpeA-bpeB* deletion was also confirmed by DNA sequencing of the PCR product obtained from KHWΔ*bpeAB* chromosomal DNA with primer AcrABpro, as described above (data not shown). The 2.3-kbp kanamycin resistance cassette from pUTKm and a 1.6-kbp fragment from *B. pseudomallei* generated by PCR with primers AcrA3′F3 and AcrB5′R3 were used as probes for the Northern blotting (Fig. 1). A 521-bp 16S ribosomal DNA PCR product, generated from KHW with primer pair 16SF2 (5′-GATGACGGTACCGGAA GAATAAGC-3′) and 16SF3 (5′-CCATGTCAAGGGTAGGTAAGGTTT-3′), was used as the probe for the 16S rRNA control.

Complementation of KHWΔ*bpeAB* mutant with wild-type *bpeAB*. A 4.9-kbp full-length *bpeAB* PCR product, amplified from KHW genomic DNA by use of the Expand Long Template PCR system (Roche Diagnostics GmbH, Mannheim, Germany) and primers AcrABpro and AcrB3′R1 (Table 2), was blunt ended with T4 DNA polymerase and was ligated with SmaI-linearized pUCP28T, yielding pUCP28T*bpeAB* (Table 1). pUCP28T*bpeAB* was first introduced into DH5αλpir by electroporation and was subsequently mobilized into KHWΔ*bpeAB* by conjugation, as described above. PCR with pUCP28T*bpeAB* isolated from complemented KHWΔ*bpeAB* and primers pUCP28TF and pUCP28TR (Table 2) produced a 5.3-kbp product which was consistent with the presence of a full-length *bpeAB* gene product (5 kb), including 300 bp of flanking plasmid DNA (data not shown).

MIC and MBC determinations. MIC determinations were carried out in 96-well microtiter plates by a standard broth microdilution method (19). Muller-Hinton broth (MHB; 5 ml; Becton Dickinson) was inoculated with 50 μl of an overnight culture, and the mixture was incubated for 4 h at 37°C with shaking. After adjustment of the culture with MHB to a 2 McFarland nephelometer standard (~5×10^8 cells/ml), the culture was further diluted 100-fold before inoculation of 10 μl into 100 μl of MHB, yielding a final inoculum density of ~5×10^5 cells/ml. Bacterial growth was determined 24 h after incubation at 35°C. The minimal bactericidal concentration (MBC) was determined by plating serially diluted cultures from the MIC test medium. The MBC was defined as the lowest concentration of antibiotic required to kill 99.9% of the inoculum. All antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Erythromycin accumulation assay. The efflux of erythromycin was studied by monitoring the intracellular levels of [^{14}C]erythromycin (NEN, Boston, Mass.) in intact cells. Overnight cultures (10 ml each) of KHW, KHWΔ*bpeAB*, and KHWΔ*bpeAB*(pUCP28T*bpeAB*) were washed three times in LB medium and were resuspended in equal volumes of fresh antibiotic-free LB medium before inoculation of 50 μl into 10 ml of antibiotic-free LB medium (optical density at 600 nm [OD_{600}], ~0.05). [^{14}C]erythromycin (final concentration, 0.1 μg/ml) was added to the culture at early log phase (OD_{600}, ~0.5), and 1-ml aliquots were removed at 30-min intervals. The cells were washed three times in 1 ml of cold 0.9% (wt/vol) NaCl containing 1 μg of erythromycin per ml, air dried, and

TABLE 2. Primers used for DNA sequencing and PCR

Primer purpose and primer	Sequence	Purpose
Sequencing of *bpeAB-oprB*		
1.	5′-CAGAAGCTTCTTGCGCATCGCGGGGCTCGT-3′	
2.	5′-GGCAATCAGTTAATATCCGTCTCT-3′	
3.	5′-CCGGGCGCGGCGAAGGTC-3′	
4.	5′-GCGCATCGAAGAGGGCGTCAAC-3′	
5.	5′-TGTGTCTCGCCGCGCTGTATGAAA-3′	
6.	5′-CGGAGCACGGCGACGAC-3′	
7.	5′-TGCAGGTCACGCAGAACACG-3′	
8.	5′-GATCTGTCGGACCTGCGCTACA-3′	
9.	5′-CAGAAGCTTCTTGCGCATCGCGGGGCTCGT-3′	
10.	5′-GGCCACCGCATCGTCGTA-3′	
11.	5′-CAAGCCCTCTTCCGCCATCAC-3′	
12.	5′-CGGCAGGCGCACGAACAG-3′	
13.	5′-CGCGGCCGGACGCTCGTAG-3′	
14.	5′-AGCGTGTTCTGCGTGACCTG-3′	
15.	5′-GCGCGATACAGGTCCACGAG-3′	
16.	5′-TTCAGCGTGCGTCTCGTGTG-3′	
PCR		
CeoA3F	5′-GACGGCGCGCTCGTGAAGAAAGG-3′	601-bp *ceoA* probe
CeoA4R	5′-TCGGCCGCCGTGTAGCCGTTGTAG-3′	
CeoB6F	5′-CAGCTCGAGCGCGGGCATCGTGTT-3′	615-bp *ceoB* probe
CeoB4R	5′-TCGGCCGCCGTGTAGCCGTTGTAG-3′	
17. AcrAHisF	5′-CGCGTCGAACGGGTTCC-3′	Full-length *bpeA* fragment
18. AcrAHisR	5′-CCCTGTTATTGCGCGCTCGA-3′	
19. AcrB3′3716	5′-ACTCGGGCCTCGTGTTCGTCA-3′	3′ *bpeB* fragment
20. AcrB3′R1	5′-CCGTGCTCCGGCTTGTCGTC-3′	
21. AcrABpro	5′-TTCCTCCTTCGTGCGTCTGGC-3′	Full-length *bpeAB* for cloning into pUCP28T
20. AcrB3′R1	5′-CCGTGCTCCGGCTTGTCGTC-3′	
AcrA3′F3	5′-GCGCATCGAAGAGGGCGTCAAC-3′	Probe for Northern blotting; hybridizes to *bpeAB* deletion in KHWΔ*bpeAB*
AcrB5′R3	5′-CAAGCCCTCTTCCGCCCATCAC-3′	
pUCP28TF	5′-GCCTGCCTTTCAGGCTGCGCAACTG-3′	Verification of *trans* complementation of KHWΔ*bpeAB*
pUCP28TR	5′-CGGGCAGTGAGCGCAACGCA-3′	

solubilized in 2 ml of scintillation cocktail (Amersham Biosciences) for liquid scintillation counting with an LS6500 multipurpose scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). The effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) on macrolide efflux was tested by adding CCCP at 20 μM (final concentration) to the culture for 10 min at 37°C prior to the addition of [^{14}C]erythromycin.

Checkerboard titration assay of pump inhibitor MC-207,110. Interactions between streptomycin, gentamicin, or erythromycin and MC-207,110 (Phe-Arg-β-naphthylamide dihydrochloride; Sigma) were assessed by a checkerboard titration assay in a 96-well microtiter plate, as described by Lomovskaya et al. (13). The antibiotics were tested at 12 twofold serial dilutions (2,048 to 0 μg/ml,), while MC-207,110 was tested at 7 twofold serial dilutions (40 to 0.625 μg/ml, including 0 μg/ml). A total of 0.2 ml of LB medium containing 5×10^5 cells/ml was added to each well, and the plate was incubated for 24 h at 37°C.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for *bpeR-bpeA-bpeB-oprB* of *B. pseudomallei* ATCC 23343 is AY325270.

RESULTS

***B. pseudomallei bpeR-bpeAB-oprB* encodes an efflux pump belonging to the RND family.** A contig of 7,597 bp containing four open reading frames (ORFs) was obtained. The ORFs were mapped to nucleotide positions 941806 to 949450 on chromosome 1 of the recently completed *B. pseudomallei* strain K96243 genome sequence (www.sanger.ac.uk). A difference between the ATCC 23343 sequence and that of the K96243 sequence was a 49-nucleotide GC-rich sequence located at positions 944037 to 944084 on the K96243 genome that is absent from strain ATCC 23343. The four ORFs are *bpeR* (636 bp), which encodes a 211-amino-acid (aa) repressor protein of the TetR family; *bpeA* (1,209 bp), which encodes a 402-aa periplasmic linker protein; *bpeB* (3,201 bp), which encodes a 1,066-aa inner membrane protein; and *oprB* (1,599 bp), which encodes a 532-aa outer membrane protein.

Several homologues of the RND family of efflux pumps, identified from a search of the National Center for Biotechnology Information protein database with the BLASTX program by using the BpeA, BpeB, OprB, and BpeR protein sequences as queries, were analyzed by multiple-amino-acid-sequence alignment. BpeA shared 54, 52, 42, and 22% amino acid similarities with AcrA (*E. coli*), MexA (*P. aeruginosa*), AmrA (*B. pseudomallei*), and CeoA (*B. cepacia*), respectively, while BpeB shared 65, 62, 49, and 40% amino acid similarities with AcrB (*E. coli*), MexB (*P. aeruginosa*), AmrB (*B. pseudomallei*), and CeoB (*B. cepacia*), respectively. The OprB

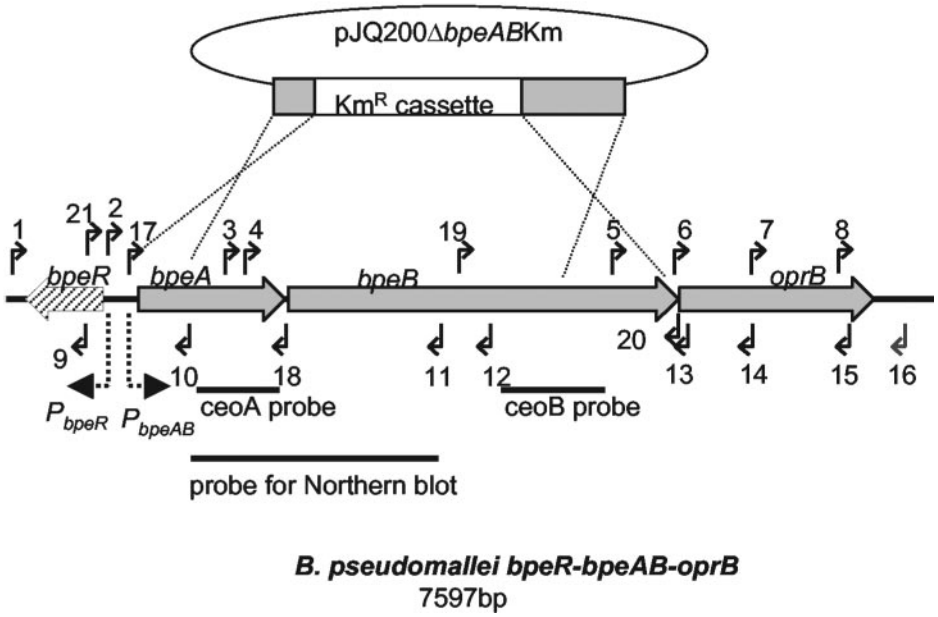

FIG. 1. Organization of *bpeR-bpeA-bpeB-oprB* genes in *B. pseudomallei* and the locations of primers for (i) sequence determination, (ii) cloning of the full-length *bpeAB* for *trans* complementation, and (iii) verification of the allelic exchange. The thin dotted lines illustrate the allelic exchange involved in the construction of KHWΔ*bpeAB*. Solid arrows and their numbers indicate the locations of the primers listed in Table 2, while the predicted promoters for *bpeAB-oprB* are represented by the thick dotted arrows. The divergently transcribed *bpeR* is indicated by the stippled arrow. The probes generated with the PCR primers listed in Table 2 are indicated as solid bars at the bottom.

protein shared 18, 52, and 28% amino acid similarities with TolC (*E. coli*), OprM (*P. aeruginosa*), and OpcM (*B. cepacia*), respectively; and BpeR shared 60, 57, and 41% amino acid similarities with AcrR (*E. coli*), AmrR (*B. pseudomallei*), and MexR (*P. aeruginosa*), respectively. These homologues belonged to the AcrAB-TolC, MexAB-OprM, AmrAB-OprA, and CeoAB-OpcM RND efflux pumps of *E. coli*, *P. aeruginosa, B. pseudomallei*, and *B. cepacia*, respectively. BpeB was also predicted to contain the conserved motifs and the characteristic structure of the inner membrane component of the RND family by having a 12-transmembrane helical domain structure with two large periplasmic loops between transmembrane segments 1-2 and 7-8 (17, 24). Putative promoter regions are located 15 and 10 bp from the start codons of *bpeA-bpeB-oprM* and *bpeR*, respectively (Fig. 1) (26).

Construction of *bpeAB* deletion mutant KHWΔ*bpeAB* and *trans* complementation with pUCP28*bpeAB*. Northern blotting was performed to verify that the *bpeAB* deletion in KHWΔ*bpeAB* resulted in a null mutation (Fig. 2). The mutation in KHWΔ*bpeAB* could be complemented in *trans* by using plasmid pUCP28T*bpeAB*, which carried the full-length *bpeA-bpeB* sequence, with restoration of *bpeAB* mRNA expression (Fig. 2). No significant increase in the level of mRNA expression was detected in the complemented mutant compared to that in the wild type, even though pUCP28T was a multicopy plasmid (Fig. 2).

Substrate specificities of the *B. pseudomallei* BpeAB-OprB efflux pump. The susceptibilities of KHW and KHWΔ*bpeAB* to a variety of antimicrobial agents were compared in an attempt to identify the substrates of the *B. pseudomallei* BpeAB-OprB pump. Table 3 summarizes the MICs and MBCs of the different antimicrobial agents for KHW, KHWΔ*bpeAB*, and *trans*-complemented mutant KHWΔ*bpeAB*(pUCP28T*bpeAB*). The parental strain, KHW, was resistant to a variety of antibiotics and agents, including aminoglycosides, macrolides, polymyxins, β-lactams, and sodium dodecyl sulfate. Intermediate resistance to DNA intercalators like ethidium bromide, crystal violet, and acriflavine was observed. Deletion of *bpeAB* resulted in about 1,000-fold increased susceptibilities to the aminoglycosides gentamicin and streptomycin and the macrolide erythromycin. KHWΔ*bpeAB* remained resistant to the aminoglycoside spectinomycin and the macrolides clarithromycin and oleandomycin, showing that there was substrate

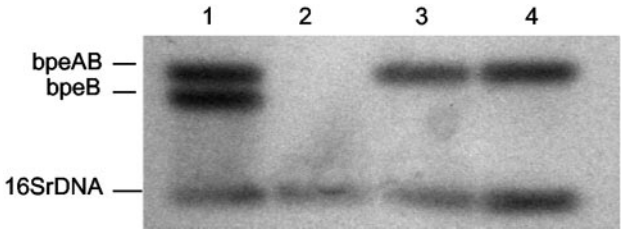

FIG. 2. Northern blot analysis of *bpeB* mRNA expression in *B. pseudomallei* KHW, KHWΔ*bpeAB*, and KHWΔ*bpeAB*(pUCP28T*bpeAB*). Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, Calif.). Total RNA (10 μg) was resolved on a 1% formaldehyde–agarose gel, transferred onto a nitrocellulose membrane, and probed with a ^{32}P-radiolabeled partial *bpeAB* PCR product generated with AcrA3′F3-AcrB5′R3 according to the instructions of the manufacturer (Amersham) (Fig. 1; Table 2). Lane 1, a mixture of 0.5 μg each of full-length *bpeAB*, *bpeB*, and 16S RNA PCR products included as a positive control; lanes 2 to 4, 10 μg each of total RNA from KHWΔ*bpeAB*, KHW, and KHWΔ*bpeAB* (pUCP28T*bpeAB*), respectively.

TABLE 3. Susceptibilities of *B. pseudomallei* KHW, KHWΔ*bpeAB,* and KHWΔ*bpeAB*(pUCP28T*bpeAB*) to antimicrobial and other agents

Antimicrobial or other agent	KHW		KHWΔ*bpeAB*		KHWΔ*bpeAB*(pUCP28T*bpeAB*)	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Aminoglycosides						
Gentamicin	128	512	0.125	0.5	256	512
Streptomycin	1,024	1,024	1	1	1,024	1,024
Spectinomycin	512	>2,000	512	>2,000	512	>2,000
Macrolides						
Erythromycin	128	1,024	0.125	0.5	>2,000	>2,000
Clarithromycin	1,024	1,024	1,024	1,024	1,024	1,024
Oleandomycin	512	>2,000	512	>2,000	512	>2,000
β-Lactams						
Cloxacillin	64	64	64	64	ND[a]	ND
Amoxicillin	>2,000	>2,000	>2,000	>2,000	ND	ND
Piperacillin	0.5	2	0.5	2	ND	ND
Ceftazidime	0.25	0.25	0.25	0.25	0.25	0.25
Cefotaxime	0.5	4	0.5	4	ND	ND
Polymyxin B	512	1,024	512	1,024	ND	ND
Tetracyclines						
Doxycycline	0.5	256	0.5	256	0.5	256
Tetracycline	1	4	1	4	1	4
Fluoroquinolones						
Ofloxacin	8	32	8	32	ND	ND
Enoxacin	1	2	ND	ND	ND	ND
Chloramphenicol	4	128	4	128	4	128
Rifampin	1	4	1	4	1	4
Novobiocin	0.5	1	0.5	1	ND	ND
Sulfodiazine	0.5	>2,000	0.5	>2,000	ND	ND
Sulfamethoxazole	128	>2,000	128	>2,000	128	>2,000
Others						
Ethidium bromide	128	128	128	128	ND	ND
Crystal violet	16	128	16	128	ND	ND
Acriflavine	16	16	4	4	16	16
SDS[b]	256	512	256	512	ND	ND

[a] ND, not determined.
[b] SDS, sodium dodecyl sulfate.

selectivity even among the aminoglycosides and macrolides. KHWΔ*bpeAB* was also more susceptible to acriflavine.

Wild-type *bpeAB* expression, with concomitant resistance to the antimicrobials gentamicin, streptomycin, erythromycin, and acriflavine, was restored in the complemented KHWΔ*bpeAB* mutant, although the level of resistance to erythromycin was significantly higher in the *trans*-complemented strain than in the wild type (Table 3). The complemented strain was also slightly more resistant to gentamicin; but its susceptibilities to spectinomycin, streptomycin, clarithromycin, oleandomycin, ceftazidime, doxycycline, tetracycline, chloramphenicol, rifampin, and acriflavine were comparable to those of the wild type. This suggests that overexpression of BpeAB-OprB is unlikely to affect the susceptibility of *B. pseudomallei* to antimicrobials of therapeutic importance for melioidosis. The successful complementation of the mutant with pUCP28T*bpeAB* also demonstrated either that the mutation in KHWΔ*bpeAB* did not have a polar effect on *oprB* or that another outer membrane efflux pump component could compensate for the OprB function.

Efflux of [^{14}C]erythromycin. A threefold higher intracellular level of [^{14}C]erythromycin (~3.0 ng/OD_{600} unit) was observed in KHWΔ*bpeAB* compared to the levels observed in KHW and the complemented KHWΔ*bpeAB* mutant (0.8 ng/OD_{600} unit) after 3 h (Fig. 3), suggesting the involvement of the BpeAB-OprB pump in the efflux of erythromycin. The ability to restore intracellular levels of erythromycin to the wild-type levels through *trans* complementation of KHWΔ*bpeAB* with full-length *bpeAB* DNA supports the role of *bpeAB* in the efflux of erythromycin. The addition of 20 μM CCCP, a proton conductor, resulted in rapid intracellular accumulation of [^{14}C]erythromycin to about 6 ng/OD_{600} unit after 3 h in both the wild type and the KHWΔ*bpeAB* mutant.

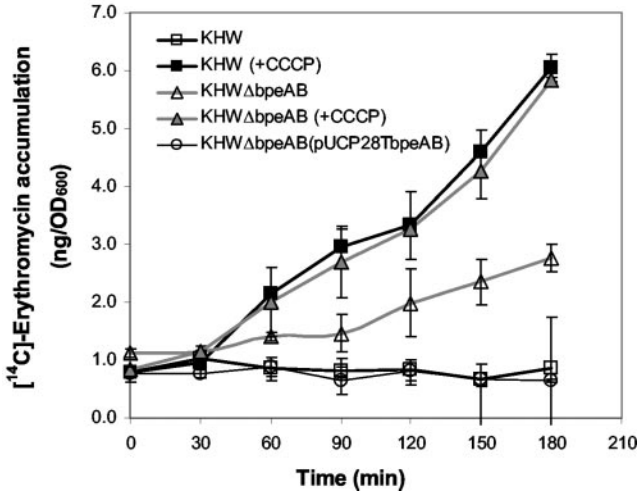

FIG. 3. Intracellular accumulation of [^{14}C]erythromycin by *B. pseudomallei* KHW, KHWΔ*bpeAB*, and KHWΔ*bpeAB*(pUCP28T*bpeAB*) in the presence and absence of 20 μM CCCP. Closed and open symbols, intracellular levels of [^{14}C]erythromycin in CCCP-treated and untreated samples, respectively. The average standard deviation of the data was 16.5%.

Effect of broad-spectrum efflux pump inhibitor MC-207,110 on BpeAB-OprB. The potentiating effect of the broad-spectrum efflux pump inhibitor MC-207,110 on the antimicrobial substrates of the *B. pseudomallei* BpeAB-OprB pump was assessed by checkerboard assays, in which the MICs of erythromycin and streptomycin were determined in the presence of different concentrations of MC-207,110 (13). In contrast to its potentiating effect on fluoroquinolones in *P. aeruginosa*, the addition of MC-207,110 at concentrations up to 40 μg/ml did not potentiate the antimicrobial activities of erythromycin and streptomycin in *B. pseudomallei*. MC-207,110 also did not have any antimicrobial activity.

DISCUSSION

The *B. pseudomallei bpeR-bpeAB-oprB* operon encodes a repressor of the TetR family (BpeR) and a three-component antimicrobial efflux system comprising a periplasmic linker protein (BpeA), an inner membrane protein (BpeB), and an outer membrane protein (OprB). Multiple-amino-acid-sequence alignments with homologues and secondary structure prediction showed that BpeAB-OprB pump belongs to the RND superfamily of transporters, which are known to have extremely broad substrate specificities. The large periplasmic loops of RND transporters, located between transmembrane segments 1,2 and 7,8, are believed to be involved in multidrug recognition and efflux (9, 15, 30).

In *B. pseudomallei*, at least one other efflux system, AmrAB-OprA, contributes to resistance to aminoglycosides and macrolides (18). Both the BpeAB-OprM pump and the AmrAB-OprA pump share some similarity with respect to substrate specificity but are distinct in their chromosomal locations and amino acid sequences. Both pumps efflux the aminoglycosides gentamicin and streptomycin and the macrolide erythromycin. We could not compare the efflux of kanamycin, which is also a substrate of the AmrAB-OprA pump, because KHWΔ*bpeAB* was kanamycin resistant. Although both pumps have some substrates in common, their substrates differed in that *B. pseudomallei* strains which had either *amrA* or *amrB* deletions displayed increased susceptibilities to a wider range of aminoglycosides and macrolides, including gentamicin, kanamycin, streptomycin, spectinomycin, tobramycin, neomycin, erythromycin, and clarithromycin (18). The *B. pseudomallei bpeAB* deletion, in contrast, resulted in increased susceptibility to erythromycin, streptomycin, and gentamicin but not to spectinomycin and clarithromycin. Although this was likely to be attributed to differences in the substrate specificities of these two pumps, it was also possible that the inactivation of the BpeAB-OprB pump in KHWΔ*bpe*AB might have consequentially upregulated the AmrAB-OprA pump, resulting in the higher levels of efflux of clarithromycin and spectinomycin from this strain. However, limited data on the inducibility of the AmrAB-OprA pump showed that it is not inducible by its substrate, and it is difficult to explain how the efflux of clarithromycin and spectinomycin could be selectively affected by this upregulation (18). A study on the effect of the *bpeAB* deletion in a strain that already lacks *amrAB* in order to elucidate whether these pumps have an additive effect on aminoglycoside and macrolide resistance in *B. pseudomallei* would be useful, since additive or multiplicative effects on drug resistance have been reported for *P. aeruginosa*, which has multiple efflux pumps with overlapping substrate specificities (12).

The data on the susceptibility of the complemented KHWΔ*bpeAB* strain to antimicrobials showed that, apart from erythromycin, multiple copies of *bpeAB-oprB* did not increase the level of resistance of *B. pseudomallei* to any of the antimicrobials tested. Coupled with the absence of any significant increase in the level of *bpeAB* mRNA expression in the complemented mutant, this would suggest that the expression of *bpeAB-oprB* is tightly regulated, perhaps by an abundance of the BpeR repressor. The successful complementation of the *bpeAB* mutation in *trans* with a plasmid carrying full-length *bpeAB* genes showed that the *bpeAB* deletion in KHWΔ*bpeAB* did not have a polar effect on *oprB* expression, or alternatively, another outer membrane efflux pump component could compensate for the absence of OprB in KHWΔ*bpeAB*. For instance, the OprM outer membrane component is shared by MexAB and MexXY in *P. aeruginosa*, and TolC is shared by AcrAB and AcrEF in *E. coli* (22).

Although *B. pseudomallei* is intrinsically resistant to a number of antibiotics, it is also highly susceptible to many others, including piperacillin, ceftazidime, tetracycline, doxycycline, and chloramphenicol; but the choice of antimicrobials for effective treatment of melioidosis remains limited. Combinations of chloramphenicol, doxycycline, and trimethoprim-sulfamethoxazole, which have been used previously to treat confirmed cases of acute severe melioidosis (3), were ineffective because of their bacteriostatic rather than bactericidal properties and their potential toxicities. At present, ceftazidime-containing regimens, imipenem, and amoxicillin-clavulanate are the preferred therapies for acute melioidosis; but the emergence of chloramphenicol- and ceftazidime-resistant strains which are fully virulent is a cause for concern (7). The use of a combination of quinolones, such as ciprofloxacin, and macrolides has also been suggested for melioidosis therapy because cipro-

floxacin could penetrate phagocytic cells and the macrolide could reduce or inhibit biofilm formation, both mechanisms of which are relevant for the treatment of melioidosis relapses (31, 32, 33). The contribution of BpeAB-OprB, as well as AmrAB-OprA, to the intrinsic resistance of *B. pseudomallei* to the antimicrobials gentamicin, streptomycin, and erythromycin would explain why aminoglycoside–β-lactam combinations, which are commonly used to treat suspected cases of community-acquired sepsis in many parts of the world, would be ineffective for the treatment of melioidosis (12, 14, 18, 20, 23).

It is noteworthy that in the complemented KHWΔ*bpeAB* mutant, which carried multiple copies of *bpeAB*, the overexpression of BpeAB-OprB did not affect the organism's susceptibilities to the antibiotics of therapeutic importance, such as chloramphenicol, doxycycline, tetracycline, and ceftazidime, although the level of resistance to erythromycin was increased in the complemented mutant (Table 3). We also attempted to determine the frequency of occurrence of spontaneous mutants overexpressing BpeAB-OprB using the single-exposure method described by Gilbert et al. (11) but were unsuccessful in obtaining any mutants on selection media containing erythromycin at greater than twice the MIC for (data not shown). Although mutants which were twice as resistant to erythromycin, gentamicin, and streptomycin occurred at a frequency of 5×10^{-8}, none of them overexpressed BpeAB-OprB in Western blots with polyclonal anti-BpeA antibodies or in promoter assays with a plasmid carrying a P_{bpeAB} promoter-*lacZ* gene fusion (data not shown).

Since multidrug efflux by gram-negative bacteria is an energy-dependent process that is driven by the proton motive force (PMF), an increase in the intracellular level of accumulation of erythromycin by *B. pseudomallei* KHW was expected when CCCP, a proton conductor which dissipates the PMF, was added (Fig. 3) (24). However, it was unexpected that KHWΔ*bpe*AB, which had a higher intracellular level of erythromycin than KHW, would also respond likewise when it was treated with CCCP. An explanation might be that erythromycin, which is a weak base ($pK_a = 8.8$), was excluded from the cytoplasm due to the pH gradient across the energized cytoplasmic membrane of KHWΔ*bpeAB*; and spontaneous influx occurred when the PMF was dissipated by CCCP, which would account for the observed increase (28). Another interpretation might be the disruption of another PMF-dependent system, such as AmrAB-OprA, which shared the same substrates with BpeAB-OprB, with the two systems perhaps functioning additively, although preliminary evidence showed that AmrAB-OprA is not sensitive to CCCP (18). It was not clear if AmrAB-OprA efficiently effluxed CCCP at the concentration used in that study. Whether AmrAB-OprA and BpeAB-OprB function additively in the efflux of aminoglycosides and macrolides in *B. pseudomallei* could be addressed by using an isogenic derivative with deletions in both *amrAB* and *bpeAB*.

The broad-spectrum efflux pump inhibitor MC-207,110, which was active against the MexAB-OprM, MexCD-OprJ, and MexEF-OprN pumps of *P. aeruginosa*, as well as the AcrAB-TolC pump of *E. coli*, did not have any effect on erythromycin or streptomycin efflux by BpeAB-OprM in *B. pseudomallei* (13). It potentiated the antimicrobial effect of the fluoroquinolone levofloxacin in *P. aeruginosa* but not that of fluoroquinolones in *B. cepacia*, a close relative of *B. pseudomallei* (O. Lomovskaya, personal communication). The possibility of identifying compounds which might inhibit BpeAB-OprB and potentiate the effects of antimicrobials, such as aminoglycosides, macrolides, and β-lactams, for the treatment of melioidosis is being investigated.

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