# The *Burkholderia pseudomallei* BpeAB-OprB Efflux Pump: Expression and Impact on Quorum Sensing and Virulence

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BpeAB-OprB is a multidrug efflux pump of the bacterial pathogen *Burkholderia pseudomallei* and is responsible for conferring antimicrobial resistance to aminoglycosides and macrolides. Expression of *bpeAB-oprB* is inducible by its substrate erythromycin and upon entry into stationary phase. BpeR, a member of the TetR family, functions as a repressor of the *bpeAB-oprB* operon. *bpeR* expression was similarly induced at stationary phase but lagged behind the induction of *bpeAB-oprB* expression. The induction of *bpeAB-oprB* expression could be advanced to the early exponential phase by exogenous addition of the *B. pseudomallei* autoinducers *N*-octanoyl-homoserine lactone (C8HSL) and *N*-decanoyl-homoserine lactone (C10HSL), suggesting that the *bpeAB-oprB* operon may be quorum regulated. On the other hand, acyl-homoserine lactone (acyl-HSL) production was undetectable in the *bpeAB*-null mutant and strains which overexpress *bpeR*. The failure of these strains to produce acyl-HSLs seemed to be at the level of synthesis of acyl-HSLs, as growth-phase-dependent expression of the autoinducer synthase BpsI was abolished in the *bpeAB*-null mutant. *bpsI* expression remained growth phase dependent in the *bpeR* mutant which had functional BpeAB-OprB. BpeAB-OprB function is likewise necessary for optimal production of quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and for biofilm formation. Cell invasion and cytotoxicity towards human lung epithelial (A549) and human macrophage (THP-1) cells were also significantly attenuated in both the *bpeAB* mutant and *bpeR*-overexpressing strains, thus suggesting the possibility of attenuating *B. pseudomallei* virulence using inhibitors of the BpeAB-OprB efflux pump.

*Burkholderia pseudomallei* is a gram-negative soil saprophyte and the etiological agent of melioidosis in humans and animals. Melioidosis is endemic in southeast Asia, tropical countries, and northern Australia. The bacterium is intrinsically resistant to penicillin, aminopenicillins, narrow-spectrum and expanded-spectrum cephalosporins, most aminoglycosides, macrolides, rifampin, and polymyxins (4). It is, however, susceptible to broad-spectrum cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone; carbapenems; chloramphenicol; tetracyclines; co-trimoxazole; and some fluoroquinolones (4). Ceftazidime is the drug of choice for the treatment of acute severe melioidosis. Prolonged maintenance therapy comprising either co-amoxiclav or co-trimoxazole and doxycycline is recommended, but relapses are common, typically occurring about 21 weeks after therapy (4).

Two multidrug efflux pumps of the resistance-nodulation-division (RND) family, AmrAB-OprA and BpeAB-OprB, have been described for *B. pseudomallei*, and both are responsible for conferring resistance to the aminoglycosides and macrolides such as gentamicin, kanamycin, streptomycin, spectinomycin, tobramycin, neomycin, erythromycin, and clarithromycin (3, 22). Although it is yet to be verified, AmrAB-OprA and BpeAB-OprB efflux pumps are believed to be regulated by repressor proteins encoded by divergently transcribed *amrR* and *bpeR*, respectively. The *bpeR* gene, located upstream of the *bpeAB-oprB* operon, encodes a repressor protein of the TetR family.

There are recent reports of a link between homoserine lactone (HSL)-mediated quorum sensing and the *mexAB-oprM* pump in *Pseudomonas aeruginosa* (17, 20, 32). *P. aeruginosa mexAB-oprM* expression was enhanced by the autoinducer *N*-butyryl-L-homoserine lactone (C4HSL), and this C4HSL-mediated enhancement of *mexAB-oprM* expression, which was independent of MexR function (32), could be negated by MexT, a positive regulator of the *mexEF-oprN* operon (20). In this study, we investigated the properties of BpeR as a repressor of the *B. pseudomallei* BpeAB-OprB efflux pump and demonstrated the interrelationship between the BpeAB-OprB efflux function, quorum sensing, and virulence. We also showed that inhibition of BpeAB-OprB could result in virulence attenuation via the inhibition of quorum sensing in *B. pseudomallei*.

## 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, the cultures were grown under aerobic conditions at 37°C in Luria-Bertani agar (LA) or Luria-Bertani broth (LB) (Becton Dickinson, Cockeysville, Md.). *B. pseudomallei* KHW is a virulent clinical isolate which we have used previously (5). Antibiotic concentrations for *Escherichia coli*, when used, were as follows: ampicillin, 50 μg/ml; gentamicin, 50 μg/ml; trimethoprim, 25 μg/ml; kanamycin, 10 μg/ml; streptomycin, 50 μg/ml; chloramphenicol, 10 μg/ml; tetracycline, 5 μg/ml. For *Agrobacterium tumefaciens* they were as follows: kanamycin, 50 μg/ml, and tetracycline, 5 μg/ml. For *B. pseudomallei* they were as follows: kanamycin, 200 μg/ml; trimethoprim, 100 μg/ml; tetracycline, 25 μg/ml. All antibiotics were purchased from Sigma (St Louis, Mo.).

**Construction of mutants and strains for complementation.** The *bpeR* mutant KHW*bpeR*::Km was derived from *B. pseudomallei* KHW by insertional mutagenesis and homologous recombination as described previously (3). The 631-bp *bpeR* product was amplified using the primers AcrRHisF (5′TCA**GGATCC**GC CAGACGCACGAAGGAGGAA3′) and AcrRHisR (5′CAG**AAGCTT**CTTGC GCATCGCGGGGCTCGT3′) and ligated with T-tailed pGEM-T (Promega, Madison, Wis.), yielding pGEM-T*bpeR*. pGEM-T*bpeR* was linearized with NarI,

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
| DH5αλpir | λpir lysogen of DH5α for replication of *ori*R6K, *ori*T, and *mob* region of RP4; Kan^s | N. Judson, Gibco-BRL |
| HB101(pRK600) | Helper strain for triparental mating; *supE44 hsdS20*(r_B m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* Cm^r | 8, 31 |
| JB525 | Derivative of *E. coli* MC1000 harboring plasmid pJBA132 Tet^r | 1 |
| *A. tumefaciens* NTI (*traR tra::lacZ749*) | pTi58-cured derivative of *A. tumefaciens* C58; contains pAtC58, pJM649, and pSVB33 | 27 |
| *B. pseudomallei* | | |
| KHW | Wild-type parental strain, clinical isolate | 5 |
| KHW*bpeR*::Km | *bpeR*::Km derivative of KHW; Kan^r | This study |
| KHW*bpeR*(pUCP28T*bpeR*) | KHW*bpeR*::Km complemented in *trans* with pUCP28T*pbeR*; Kan^r Tmp^r | This study |
| KHWΔ*bpeAB* | KHWΔ*bpeAB* Kan^r | 3 |
| KHWΔ*bpeAB*(pUCP28Tbpe*AB*) | KHWΔ*bpeAB* complemented in *trans* with pUCP28Tbpe*AB*; Kan^r Tmp^r | 3 |
| **Plasmids** | | |
| pGEM-T | Vector for PCR cloning; Amp^r | Promega |
| pGEM-T*bpeR* | pGEM-T carrying the 631-bp *bpeR* PCR product obtained with AcrRHisF and AcrRHisR primers | This study |
| pGEM-T*bpeR*::Km | pGEM-T*bpeR* carrying a 2.3-kbp kanamycin-resistance cassette from pUTKm | This study |
| pJQ200mp18 | Mobilizable allelic-exchange vector; *traJ sacB* Gen^r | 29 |
| pJQ200*bpeR*::Km | pJQ200mp18 carrying a 3-kbp Apal-Spel fragment containing *bpeR* and kanamycin resistance cassette | This study |
| pUCP28T | Broad-host range vector; *inc*P *ori*T, pRO1600 *ori* Tmp^r | 39 |
| pUCP28T*bpeR* | pUCP28T carrying 1.5-kb *bpeR* PCR product containing *bpeR* promoter; Tmp^r | This study |
| pUCP28T*bpeAB* | pUCP28T carrying 4.9-kbp *bpeAB* PCR product containing *bpeAB* promoter; Tmp^r | 3 |
| pUTKm | Source of kanamycin resistance cassette; *ori*R6K *mob*RP4 Kan^r Amp^r | 7 |
| pMC1403 | pBR322-derived plasmid; source of promoterless *lacZYA* fragment; Amp^r | 2 |
| Mini-CTX1 | Broad-host-range plasmid; *ori*T Tet^r | 15 |
| pCYY | Mini-CTX1 carrying promoter less *lacZYA* cassette from pMC1403 | This study |
| pCYY*bpeAB* | pCYY ligated to *bpeAB* promoter fragment | This study |
| pCYY*bpeR* | pCYY ligated to *bpeR* promoter fragment | This study |
| pSY1 | pCYY carrying 1.2-kbp *bpsI*_promoter-*lacZ* gene fusion; Tet^r | 34 |

[a] Kan, kanamycin; Tmp, trimethoprim; Gen, gentamicin; Tet, tetracycline; Cm, chloramphenicol; Amp, ampicillin.

made blunt ended, and ligated with a 2.3-kbp end-filled EcoRI fragment carrying the kanamycin resistance cassette from pUTKm, yielding pGEM-T*bpeR*::Km. Underlined sequences refer to restriction endonuclease recognition sites. T4 DNA polymerase (Promega) was used to generate blunt ends in DNA fragments with 3′ or 5′ overhanging ends. pGEM-T*bpeR*::Km was then digested with ApaI and SpeI, and the 3-kbp ApaI-SpeI fragment carrying *bpeR* and the kanamycin resistance cassette from pGEM-T*bpeR*::Km was isolated and made blunt ended before being inserted into SmaI-linearized pJQ200mp18, yielding pJQ200*bpeR*::Km. pJQ200*bpeR*::Km was first introduced into *E. coli* DH5αλpir by electroporation using a MicroPulser (Bio-Rad, Hercules, CA) and then mobilized into *B. pseudomallei* KHW by triparental mating using *E. coli* HB101(pRK600) as a helper strain (7). Recombinants which had undergone allelic exchange of *bpeR* were selected on LA containing kanamycin, streptomycin, and 5% (wt/vol) sucrose and were designated KHW*bpeR*::Km (Table 1). The *bpeR*-null mutation was verified by PCR and reverse transcription-PCR (RT-PCR) using primers AcrRHisF and AcrRHisR. PCR yielded a 631-bp fragment from KHW but a 3-kbp fragment from KHW*bpeR*::Km, which was consistent with the disruption of *bpeR* by a 2.3-kbp kanamycin resistance cassette (data not shown). RT-PCR using primer pair AcrAHisF (5′TCAGGATC CCGCGTCGAACGGGTTCC3′) and AcrAHisR (5′CAGAAGCTTCCTGTTAT TGCGCGCTCG3′) was applied to detect *bpeAB* expression, and AcrRHisF and AcrRHisR were used to detect *bpeR* expression (Fig. 1). RT-PCR of 16S RNA using primers 16SF2 (5′GATGACGGTACCGGAAGAATAAGC3′) and 16SR2 (5′CC
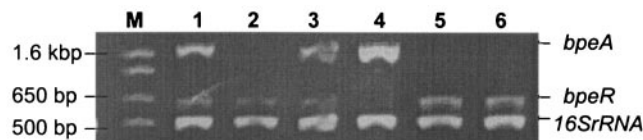


FIG. 1. Analyses of *bpeA* and *bpeR* expression by RT-PCR. RT-PCR results obtained using total RNA isolated from exponential-phase cultures of wild-type parental strain KHW (lane 1), KHWΔ*bpeAB* mutant (lane 2), KHWΔ*bpeAB*(pUCP28T*bpeAB*) (lane 3), KHW*bpeR*::Km (lane 4), KHW*bpeR*::Km(pUCP28T*bpeR*) (lane 5), and KHW(pUCP28T*bpeR*) (lane 6). Lane M is 1-kb Plus size markers (Invitrogen, Carlsbad, CA). The bands corresponding to *bpeA*, *bpeR*, and 16S rRNA RT-PCR products are indicated on the right. *bpeA* expression was absent in KHWΔ*bpeAB* (lane 2) but was restored by complementation (lane 3). *bpeR* expression was absent in KHW*bpeR*::Km (lane 4), and complementation with pUCP28T*bpeR* resulted in complete repression of *bpeA* expression (lane 5). Overexpression of *bpeR* in KHW also resulted in complete repression of *bpeA* expression (lane 6).

ATGTCAAGGGTAGGTAAGGTTT3′) was included as an internal standard for the amount of template RNA used.

A 1.5-kb full-length *bpeR* PCR product inclusive of the putative *bpeR* promoter was amplified from KHW genomic DNA using the primers AcrRHisF and AcrA5′R (5′GGCCACCGCATCGTCGTA3′). The PCR product was first converted to blunt ends using T4 DNA polymerase before ligation with SmaI-linearized pUCP28T to yield pUCP28T*bpeR* (Table 1). pUCP28T*bpeR* was mobilized into KHW*bpeR*::Km via triparental mating as described previously. PCR using DNA, and RT-PCR using total RNA, isolated from the complemented mutant confirmed the successful transfer of pUCP28T*bpeR* into KHW*bpeR*::Km (data not shown).

**Construction of promoter-*lacZ* fusions.** The broad-host-range vector pCYY, carrying a promoterless *lacZ* cassette, was used to construct *bpeR*$_{promoter}$-*lacZ* and *bpeAB*$_{promoter}$-*lacZ* fusions. pCYY was derived by ligating a 6.2-kbp EcoRI-SalI promoterless *lacZYA* cassette from pMC1403 with EcoRI-SalI-linearized mini-CTX1. An 868-bp intergenic fragment, comprising the upstream regulatory sequences of both *bpeR* and *bpeAB*, as well as the ATG initiation codons, was amplified from KHW DNA by PCR using the primers AcrABpro (5′TTCCTC CTTCGTGCGTCTGGC3′) and AcrA5′R. Opposite orientations of the blunt-ended PCR product when ligated to SmaI-linearized pCYY yielded either the *bpeAB*$_{promoter}$-*lacZ* fusion (pCYY*bpeAB*) or the *bpeR*$_{promoter}$-*lacZ* fusion (pCYY*bpeR*). The orientations of the inserts were verified by restriction digests (data not shown). Transcriptional expression of *bpeAB* and *bpeR* was assayed by measuring β-galactosidase activities in *B. pseudomallei* strains harboring these reporter plasmids.

**DNA and RNA manipulations.** Bacterial genomic DNA was isolated according to the method described by Pitcher et al. (28). Total RNA was extracted from bacteria using the RNeasy Mini Kit (Qiagen, Hilden, Germany) after pretreatment with RNAprotect reagent (Qiagen). PCR was performed in a PTC-100 Peltier thermal cycler (MJ Research, MA) in Mg$^{2+}$-free buffer containing 100 ng template DNA, 200 μmol (each) deoxynucleoside triphosphates, 50 pmol of each primer, 1.5 mM MgSO$_4$, and 0.5 U *Tth* polymerase (Biotools, Madrid, Spain) in a total volume of 50 μl. Cycling parameters include 1 cycle (3 min, 94°C) followed by 30 cycles (30 s, 94°C; 30 s of annealing at the respective temperatures; 1 min/kb of product length, 72°C) and a final extension at 72°C for 10 min. RT-PCR was carried out in 10 μl Mg$^{2+}$-free buffer containing 5 μg template RNA, 200 μM (each) deoxynucleoside triphosphates, 1 μM of each primer, 1 mM MgSO$_4$, 0.1 U *Tfl* DNA polymerase, and 0.1 U avian myeloblastosis virus reverse transcriptase (Access RT-PCR System; Promega). Cycling parameters include a reverse transcription step at 48°C for 45 min, followed by PCR comprising 1 cycle (2 min, 94°C), 30 cycles (30 s, 94°C; 30 s of annealing at the respective temperatures; 1 min/kb of product length, 68°C), and a final extension at 68°C for 7 min.

**Bioassays.** The β-galactosidase assay was performed as described by Miller (21). Briefly, 10 ml AB medium (6) containing either 20 mM glycerol or 20 mM glucose, 0.2% (wt/vol) Casamino Acids, and 25 μg/ml tetracycline was inoculated (1:100) with an overnight culture of *B. pseudomallei* KHW or its isogenic mutants, harboring either pCYY*bpeAB*, pCYY*bpeR*, or the *bpsI*$_{promoter}$-*lacZ* reporter pSYI. β-Galactosidase activities and cell densities (optical densities at 600 nm [OD$_{600}$]) were determined at various time intervals during the culture. An 0.1-ml amount of bacterial culture was used for the β-galactosidase assay. Enzyme activity was expressed in Miller units. For assays involving the addition of exogenous compounds, typically a 5-ml bacterial culture was inoculated (1:50) with overnight culture and the compounds to the culture at an OD$_{600}$ of ~0.1. *N*-Octanoyl-L-homoserine lactone (C8HSL) and *N*-decanoyl-L-homoserine lactone (C10HSL) were purchased from Sigma. β-Galactosidase activities were determined after 4 h of incubation at 37°C, i.e., in early exponential growth phase.

Siderophore production was determined using the chrome azurol assay described by Yang et al. (40). Briefly, 0.1 ml of 24-h-old bacterial culture was added to 0.9 ml chrome azurol solution (pH 5.6) and equilibrated for 2 h before absorbance was read at 630 nm. Siderophore activity was expressed as the change in OD$_{630}$ readings (ΔOD$_{630}$) between the test samples and the sample blank and was normalized for cell density by being expressed as a ratio of ΔOD$_{630}$ to OD$_{600}$.

Phospholipase C (PLC) activity was determined by the enzymatic hydrolysis of *p*-nitrophenylphosphorylcholine (NPPC; Sigma) to phosphatidylcholine and *p*-nitrophenol (18). Briefly, 10 μl of bacterial culture supernatant was mixed with 90 μl of NPPC reagent (250 mM Tris-HCl, pH 7.2; 0.1 mM ZnSO$_4$; 10 mM NPPC; 40% [wt/wt] sorbitol), and the release of *p*-nitrophenol was detected by reading OD$_{405}$ after 1 h of incubation at 37°C. The PLC activities shown have been normalized for cell density by being expressed as a ratio of OD$_{410}$/OD$_{600}$.

All assays described were performed in triplicate.

**Biofilm formation.** Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well polyvinyl chloride microtiter plates using a modification of the protocol described by O'Toole and Kolter (24). Briefly, 100 μl of a diluted (OD$_{600}$ of ~0.05) overnight bacterial culture in AB medium containing 0.2% Casamino Acids and 20 mM glycerol was added into each well of a 96-well microtiter plate. After 20 h of incubation at 30°C and being washed twice with distilled water to remove planktonic cells, 125 μl of 1% (wt/vol) crystal violet (Sigma) was added. After 15 min at room temperature and three rounds of careful washing with distilled water, 300 μl of 95% (vol/vol) ethanol was added to solubilize the stain and the extent of biofilm formation was determined by reading the absorbance of the solution at 595 nm. The assay was performed in triplicate.

**MIC and MBC determinations.** MIC determinations were carried out in 96-well microtiter plates by a standard broth microdilution method as described previously (3, 23). The minimal bactericidal concentration (MBC) was defined as the lowest concentration of antibiotic required to kill 99.9% of the inoculum. All antibiotics were purchased from Sigma Co.

**Detection of autoinducer production.** The production of acyl-homoserine lactones by the *B. pseudomallei* strains was detected by cross-streaking against an indicator strain, *E. coli* JB525 or *A. tumefaciens* NTI as described previously (34). The bacteria were cross-streaked onto LB agar and observed after incubation at 28°C for 20 h. For detection using *A. tumefaciens* NTI, the plates were overlaid with 20 μl X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 10 mg/ml) before streaking (30).

**Cell invasion and cytotoxicity assays.** Bacterial invasion of A549 and THP-1 cells was performed as described by Elsinghorst except for the following modifications (10). Tetracycline (50 μg/ml) was added to kill extracellular bacteria instead of gentamicin, as KHW is resistant to gentamicin. Mid-log-phase bacteria in LB medium (OD$_{600}$ of 0.6) were washed and resuspended in an equal volume of 0.85% (wt/vol) NaCl. Twenty-five-microliter aliquots of the bacterial suspension were added to each well of a 24-well tissue culture plate containing $1 \times 10^5$ mammalian cells per well (multiplicity of infection = 100). After 2 h of incubation at 37°C in the presence of 5% CO$_2$, the cells in each well were washed three times with phosphate-buffered saline and 1.5 ml of fresh culture medium containing tetracycline (50 μg/ml) was added. After a further 2 h of incubation to kill extracellular bacteria, the wells were again washed three times with phosphate-buffered saline and 1 ml of 0.1% Triton X-100 (Sigma) was added to lyse the mammalian cells. Serial dilutions of the cell lysates were then plated on LA to determine the number of bacteria in the cells after a 2-h exposure. The assays were performed in triplicate. When C8HSL was used, it was added to the mammalian cell culture medium to a final concentration of 100 nM together with the addition of 25 μl of bacterial suspension.

The cytotoxic effects of bacteria on mammalian cells were evaluated by measuring the release of lactate dehydrogenase enzyme using a Cytotoxicity Detection kit (Roche, Mannheim, Germany). Mid-log-phase bacterial cells were added to A549 and THP-1 cells (10$^5$ cells/well) cultured in 24-well plates in Dulbecco's modified Eagle's medium (Sigma) and RPMI 1640 (Sigma), respectively, each supplemented with 10% (vol/vol) fetal bovine serum (Sigma). The multiplicity of infection was 100. After 1 h of incubation at 37°C in the presence of 5% CO$_2$, tetracycline (50 μg/ml) was added and the cells were further incubated for 4 h. A 100-μl aliquot of the centrifuged supernatant obtained from each well was used for the lactate dehydrogenase assay. The assays were performed in triplicate. When C8HSL was used, it was added to the mammalian cell culture medium to a final concentration of 100 nM together with 25 μl of bacterial suspension.

## RESULTS

**BpeR acts as a repressor of the BpeAB-OprB multidrug efflux pump.** BpeR, a member of the TetR repressor family, shared significant similarities in amino acid sequence with MexR of *P. aeruginosa* (3). We constructed a *bpeR*-null mutant, KHW*bpeR*::Km, by insertional mutagenesis. The *bpeR*-null mutation was verified by RT-PCR and is associated with a corresponding increase in *bpeA* expression (Fig. 1). Expression of *bpeR* was restored when KHW*bpeR*::Km was complemented in *trans* using pUCP28T*bpeR* (Fig. 1). Similarly, the expression of *bpeA*, which was lacking in the mutant KHWΔ*bpeAB*, could also be restored by complementation with pUCP28T*bpeAB* (Fig. 1). Additionally, *bpeA* expression was completely re-

TABLE 2. Antimicrobial susceptibilities of *B. pseudomallei* KHW, KHWΔ*bpeAB*, KHW*bpeR*::Km, and the complemented mutants KHW*bpeR*::Km(pUCP28T-*bpeR*) and KHW(pUCP28T-*bpeR*)

| Antimicrobial agent | KHW | | KHWΔ*bpeAB* | | KHW*bpeR*::Km | | KHW*bpeR*::Km(pUCP28T*bpeR*) | | KHW(pUCP28T*bpeR*) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC (μg/ml) | MBC (μg/ml) | MIC (μg/ml) | MBC (μg/ml) | MIC (μg/ml) | MBC (μg/ml) | MIC (μg/ml) | MBC (μg/ml) | MIC (μg/ml) | MBC (μg/ml) |
| Gentamicin | 128 | 256 | 0.125 | 0.5 | 2,048 | 2,048 | 1 | 4 | 1 | 4 |
| Streptomycin | 1,024 | 1,024 | 1 | 1 | 2,048 | 2,048 | 1 | 32 | 1 | 32 |
| Erythromycin | 128 | 1,024 | 0.125 | 0.5 | 512 | 2,048 | 4 | 1,024 | 64 | 1,024 |

pressed when *bpeR* was overexpressed in both the wild-type strain, KHW(pUCP28T*bpeR*), and in the *bpeR* mutant KHW*bpeR*::Km(pUCP28T*bpeR*). The effect of *bpeR* overexpression was the same as that of the *bpeAB*-null mutation (Fig. 1).

We next determined the MICs and MBCs of antibiotics that are substrates of the BpeAB-OprB efflux pump in wild-type KHW, KHWΔ*bpeAB*, and KHW*bpeR*::Km, as well as the mutants which have been complemented in *trans* using pUCP28T*bpeAB* and pUCP28T*bpeR*, respectively. Previously, we showed that gentamicin, streptomycin, and erythromycin are substrates of the BpeAB-OprB pump (3). Compared to the wild-type parental strain, the *bpeR* mutant was 2-, 4-, and 16-fold more resistant to streptomycin, erythromycin, and gentamicin, respectively (Table 2). In contrast, overexpression of *bpeR* in both KHW and KHW*bpeR*::Km had the same effect of increasing susceptibility to all three antibiotics. The MICs and MBCs of all three antibiotics on KHW*bpeR*::Km(pUCP28T*bpeR*) and KHW(pUCP28T*bpeR*) were equivalent to that of KHWΔ*bpeAB* (Table 2). These data correlated well with the observed down-regulation of *bpeA* expression in the complemented *bpeR* mutant in Fig. 1. We have thus shown that overexpression of *bpeR* resulted in a significant increase in susceptibility to gentamicin, streptomycin, and erythromycin, while the *bpeR*-null mutation had the opposite effect of increasing resistance to the antibiotics. The restoration of BpeR repressor function by complementation also verified that the increased antimicrobial resistance of KHW*bpeR*::Km was indeed a consequence of the *bpeR* mutation and not due to polar effects on downstream genes or a secondary site mutation.

**BpeAB-OprB is an inducible efflux system.** We studied the expression of *bpeAB*$_{\text{promoter-lacZ}}$ in wild type and the *bpeR* mutant by introducing the plasmid pCYY*bpeAB* into KHW and KHW*bpeR*::Km, respectively. Using a time course promoter assay, we demonstrate that basal expression of *bpeAB*$_{\text{promoter-lacZ}}$ in wild type was low but was inducible almost immediately upon exposure to 0.1× MIC (or 10 μg/ml) of erythromycin (Fig. 2A). The induction of *bpeAB* expression by erythromycin showed a linear dose-dependent relationship with respect to erythromycin (Fig. 2B). As predicted, basal expression of *bpeAB* was 2.5-fold higher in KHW*bpeR*::Km than in KHW. However, the higher basal *bpeAB* expression in KHW*bpeR*::Km was still inducible in the presence of 0.1× MIC of erythromycin, and it increased to a final level similar to that obtained when erythromycin was added to the culture of KHW (Fig. 2A). We could also induce the expression of *bpeR*$_{\text{promoter-lacZ}}$ in the presence of 0.1× MIC of erythromycin using KHW(pCYY*bpeR*), but unlike *bpeAB*$_{\text{promoter-lacZ}}$ expres-

sion, the induction of *bpeR*$_{\text{promoter-lacZ}}$ expression was delayed, occurring only after 6 h of exposure to erythromycin (data not shown). Our data thus provide evidence that the BpeR repressor interacts with the *bpeAB-oprB* regulatory region to regulate *bpeAB* expression. Induction of *bpeR*$_{\text{promoter-lacZ}}$ expression in wild-type cells by erythromycin is also suggestive of a protein-ligand interaction between the BpeR repressor and erythromycin which releases the repression of *bpeAB-oprB* expression as well as *bpeR* expression. It appears that an adjacent site, which may be responsive to a different ligand-sensitive regulatory protein, may also be involved in regulating the inducible expression of the *bpeAB-oprB* operon. This yet unknown BpeR-independent mechanism could explain why, in the *bpeR* mutant background, *bpeAB* expression was elevated but not constitutively maximal (Fig. 2A).

**Expression of *bpeAB-oprB* is growth phase dependent while expression of *bpeR* is inducible upon entry into stationary phase.** We next studied the expressions of *bpeAB* and *bpeR* at different growth phases of the bacterium using KHW (pCYY*bpeAB*) and KHW(pCYY*bpeR*), respectively. The expression of *bpeAB*$_{\text{promoter-lacZ}}$ was low from the onset of culture until mid-log phase but then gradually increased to reach maximum at stationary phase (Fig. 3A). In comparison, *bpeR*$_{\text{promoter-lacZ}}$ expression was low from the onset of culture until late log phase. Induction of *bpeR*$_{\text{promoter-lacZ}}$ expression began during the early stationary phase and reached maximum during the late stationary phase (Fig. 3B). These data were further confirmed by RT-PCR, which showed undetectable expression of *bpeR* up to 12 h of culture, followed by maximum expression after 24 h of culture. The detection of the *bpeR* transcripts at 12 h coincides with entry of the culture into stationary phase. In contrast, *bpeAB* transcripts were detectable from the onset of culture but started to increase gradually from 12 h onwards and reached maximum in stationary phase (Fig. 3C).

**Addition of C8HSL and C10HSL autoinducers advances *bpeAB*$_{\text{promoter-lacZ}}$ expression to early exponential phase.** Since quorum sensing regulates the cell density-dependent expression of many bacterial genes, we then investigated if the expression of *bpeAB* and *bpeR* might be regulated by autoinducers. The *B. pseudomallei* quorum-sensing systems produce several autoinducers during stationary-phase cultures, including C8HSL and C10HSL (34, 37, 38). We wanted to know if the expression of *bpeAB*$_{\text{promoter-lacZ}}$ in wild-type KHW could be advanced to early exponential phase (8 to 12 h) by the addition of cognate autoinducers. C8HSL (100 nM) or C10HSL (100 nM) was added to KHW(pCYY*bpeAB*) cultured in AB medium containing glucose and Casamino Acids. Max-
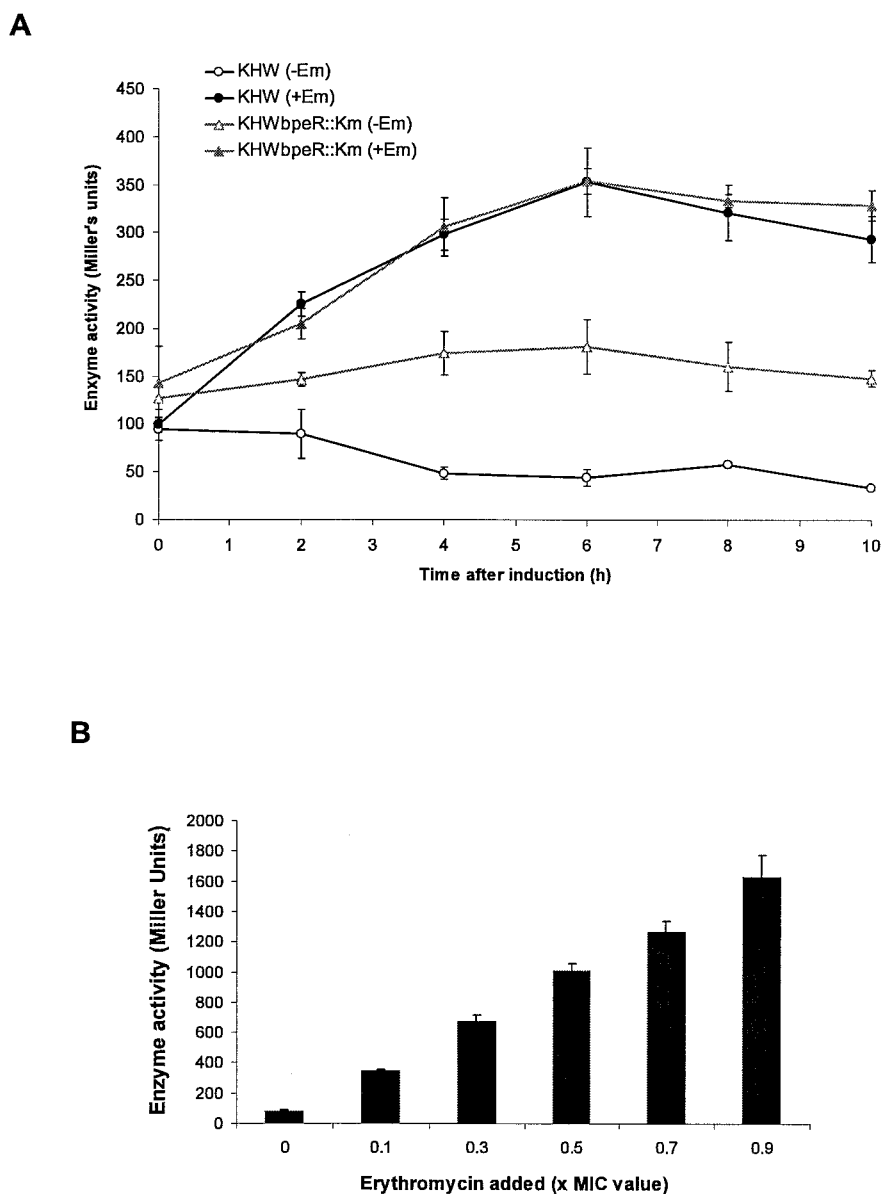
**A**



**B**



FIG. 2. Effect of erythromycin on expression of $bpeAB_{promoter}$-$lacZ$ in wild-type *B. pseudomallei* and the *bpeR*-null mutant. (A) Effect of *bpeR* mutation on the basal and inducible expression of *bpeAB* in KHW and KHW*bpeR*::Km. β-Galactosidase activities expressed from $bpeAB_{promoter}$-$lacZ$ fusion were determined in KHW (circles) and KHW*bpeR*::Km (triangles) harboring the reporter plasmid pCYY*bpeAB*. Open symbols represent basal expression of *bpeAB*, while closed symbols represent *bpeAB* expression after addition of 0.1× MIC of erythromycin (or 10 μg/ml). (B) Dose-dependent induction of *bpeAB* expression in KHW(pCYY*bpeAB*) by erythromycin. β-Galactosidase activities were assayed 4 h after the addition of erythromycin to the bacterial cultures in AB medium containing 20 mM glucose, 0.2% Casamino Acids, and 25 μg/ml tetracycline. Error bars represent standard deviations of triplicate β-galactosidase determinations for one typical experiment. Where error bars are not shown, the standard deviation was within the size of the symbol.

imum expression of $bpeAB_{promoter-lacZ}$ typically attained during stationary-phase culture (24 h) could be prematurely advanced to 12 h by the addition of C8HSL (Fig. 4A). Addition of C10HSL was less effective in advancing the expression of $bpeAB_{promoter-lacZ}$ (Fig. 4B). This suggests that *bpeAB-oprB* expression might be regulated by quorum sensing and these autoinducers might be effluxed by BpeAB-OprB. The advancement of $bpeAB_{promoter-lacZ}$ expression in KHW to early exponential phase (4 h) was also dependent on the dose of C8HSL added and required at least 80 nM C8HSL to achieve the level

of $bpeAB_{promoter-lacZ}$ expression at stationary phase (~250 Miller units) (Fig. 4C). $bpeAB_{promoter-lacZ}$ expression could not be induced if the concentration of C8HSL was <60 nM (Fig. 4C). This is in contrast to the very low concentrations of C8HSL (0.1 nM) and C10HSL (1 nM), which are required to induce the *B. pseudomallei luxI* homolog *bpsI* (34).

**BpeAB-OprB is required for autoinducer production.** There is also evidence suggesting that multidrug efflux pumps may be involved in the efflux of autoinducers (12, 26). We cross-streaked *B. pseudomallei* against the reporter strains, *E. coli*
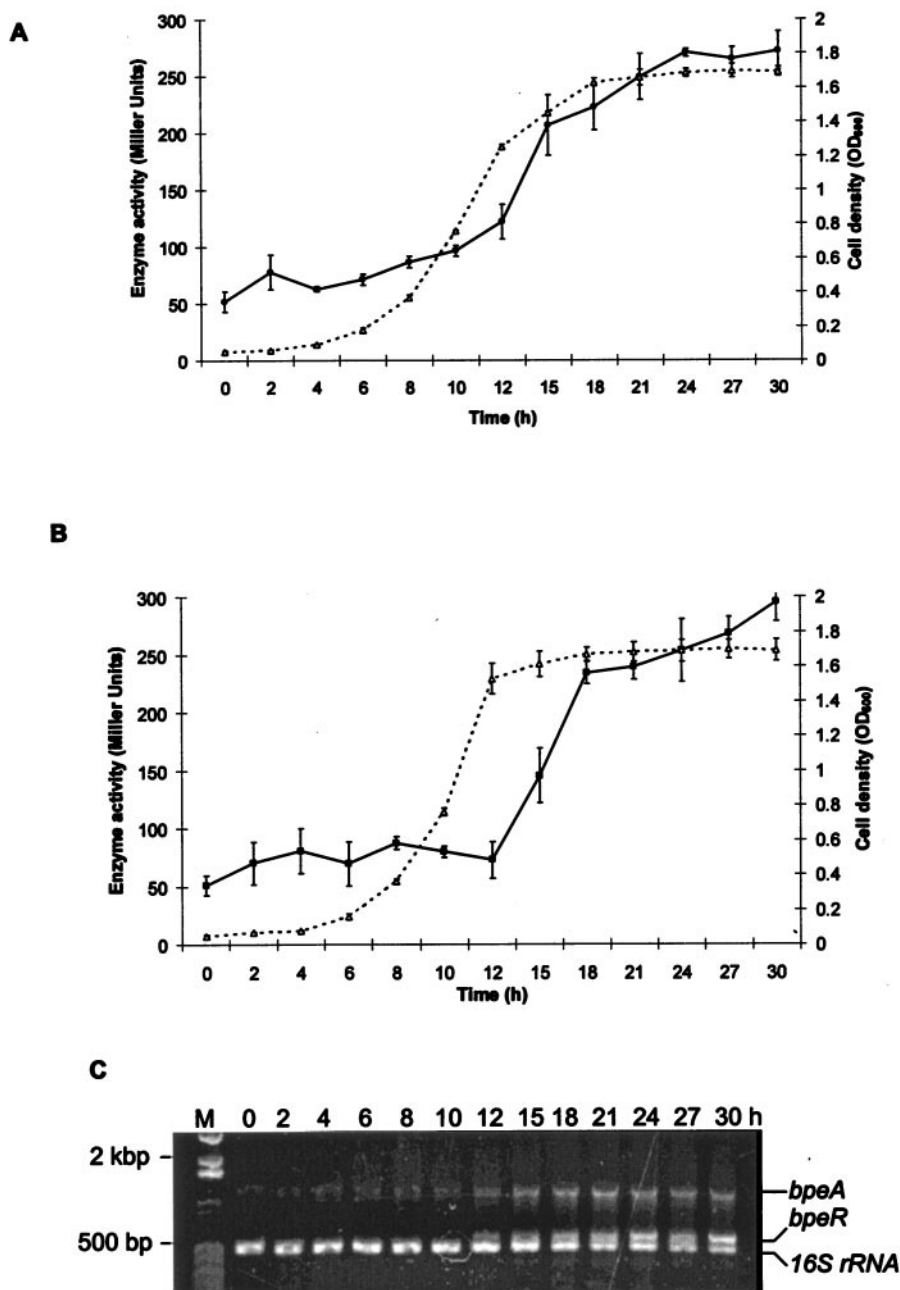
FIG. 3. Growth-phase-dependent expression of *bpeAB* and *bpeR* in wild-type KHW. (A) β-Galactosidase activities were expressed from pCYY*bpeAB* in KHW. (B) β-Galactosidase activities were expressed from pCYY*bpeR* in KHW. Closed circles or squares represent β-galactosidase activities in Miller units, while open triangles represent cell densities (OD$_{600}$). Error bars represent standard deviations of triplicate determinations of cell densities and β-galactosidase activities. Where error bars are not shown, the standard deviation was within the size of the symbol. The bacteria were cultured in AB medium containing 25 μg/ml tetracycline, 20 mM glycerol, and 0.2% (wt/vol) Casamino Acids. (C) Verification of growth-phase-dependent expression of *bpeA* and *bpeR* in wild-type cells by RT-PCR. RT-PCR was performed to detect *bpeA* and *bpeR* expression from the onset of culture (0 h) to stationary phase (30 h) (upper row). RT-PCR of 16S rRNA was included as an internal control for the reaction and to normalize the amount of total RNA used. Bands corresponding to the *bpeA*, *bpeR*, and 16S rRNA transcripts are indicated on the right, while lane M is the 1-kb Plus molecular size markers.

JB525 and *A. tumefaciens* NTI. Autoinducers produced by KHW were detected by the appearance of green fluorescence on the *E. coli* JB525 reporter streak (Fig. 5A, panel I). Cross-streaks of KHWΔ*bpeAB* against the *E. coli* JB525 reporter failed to yield any green fluorescence (Fig. 5A, panel II). Au-

toinducer production was restored in the complemented *bpeAB* mutant, KHWΔ*bpeAB* (pUCP28T*bpeAB*), and in the *bpeR* mutant, KHW*bpeR*::Km, which showed that *bpeAB-oprB* expression was necessary for this process (Fig. 5A, panels III and IV). Additionally, the absence of autoinducer production
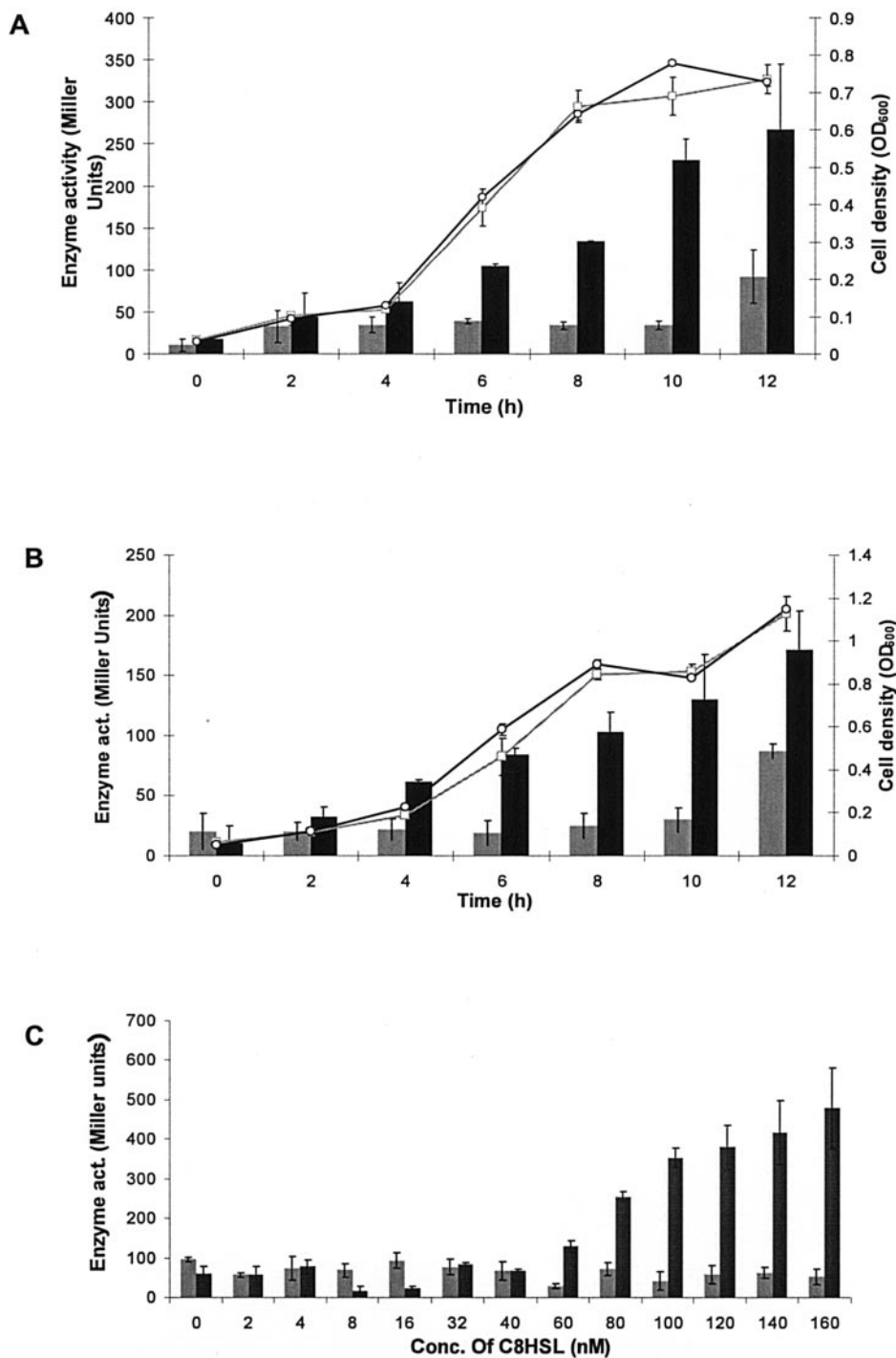
FIG. 4. Advancement of growth-phase-dependent induction of *bpeAB-lacZ* expression to early exponential phase by exogenous C8HSL and C10HSL. (A) Growth curves ($OD_{600}$) of KHW in the presence (○) or absence (□) of 100 nM C8HSL. Also shown are the β-galactosidase activities (Miller units) representing *bpeAB* expression from pCYYbpeAB from onset of culture (0 h) to early exponential phase (12 h) in the absence (grey bars) or presence (black bars) of C8HSL, respectively. (B) Growth curves ($OD_{600}$) of KHW in the presence (○) or absence (□) of 100 nM C10HSL. Also shown are β-galactosidase activities (Miller units) representing *bpeAB* expression in the absence (grey bars) or presence (black bars) of C10HSL, respectively. (C) Dose-dependent induction of *bpeAB-lacZ* expression by exogenous C8HSL. The horizontal axis represents the concentrations of exogenous C8HSL added to the culture medium. Black bars represent cultures to which different amounts of C8HSL were added, while grey bars represent control cultures to which no C8HSL was added. C8HSL was added to the bacterial cultures in AB medium containing 20 mM glucose, 0.2% Casamino Acids, and 25 μg/ml tetracycline about 1 h after inoculation from an overnight culture ($OD_{600}$ of ~0.1 to 0.2), and β-galactosidase assays were performed on aliquots of the cultures 4 h after the addition of exogenous C8HSL. Error bars represent standard deviations of triplicate β-galactosidase determinations for one typical experiment.
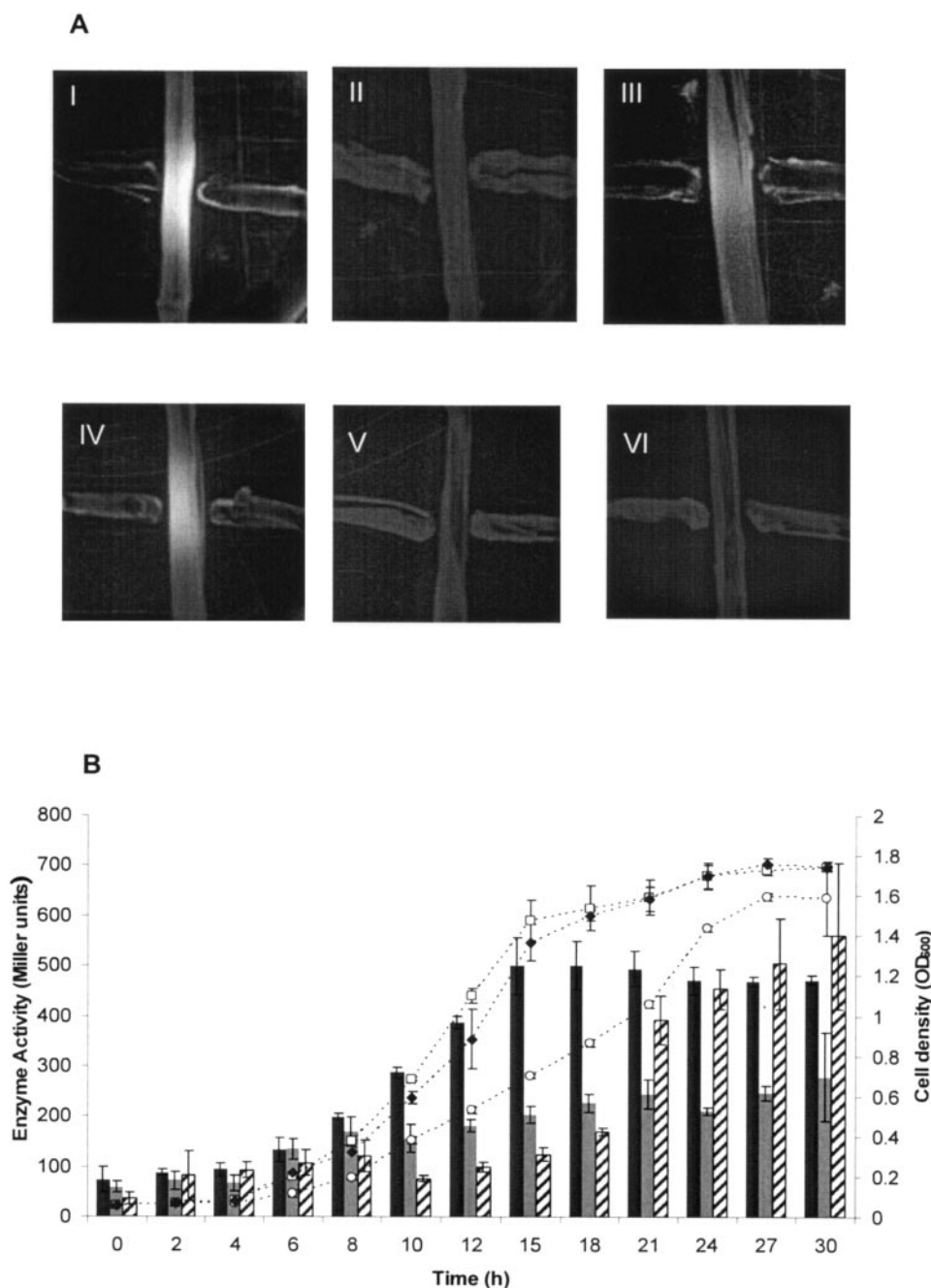
**A**



**B**



FIG. 5. (A) Detection of autoinducer production by *B. pseudomallei* KHW and its derivatives. Vertical streaks represent the *E. coli* JB525 reporter strain harboring the *luxR-luxI*$_{promoter}$-GFP plasmid (pJBA132), while the horizontal streaks represent (I) KHW, (II) KHWΔ*bpeAB* mutant, (III) KHWΔ*bpeAB*(pUCP28T-*bpeAB*)-complemented mutant, (IV) KHW*bpeR*::Km mutant, (V) KHW*bpeR*::Km(pUCP28T-*bpeR*)-complemented mutant, and (VI) KHW(pUCP28T*bpeR*), respectively. Fluorescence on the vertical streaks indicates the activity of autoinducers produced by the horizontal streaks of tester strains. (B) Effect of BpeAB-OprB on growth-phase-dependent expression of *bpsI*$_{promoter-lacZ}$. The plasmid pSYI carrying *bpsI*$_{promoter}$-*lacZ* was introduced into KHW, KHWΔ*bpeAB*, and KHW*bpeR*::Km, respectively, to study the effect of BpeAB-OprB on the expression of the autoinducer synthase BpsI. Aliquots of the bacteria cultured in AB medium containing 20 mM glycerol, 0.2% Casamino Acids, and 25 μg/ml tetracycline were assayed at different time intervals from the onset of culture (0 h) to stationary phase (30 h). Dotted lines represent the cell densities of KHW(pSYI) (□), KHWΔ*bpeAB*(pSYI) (○), and KHW*bpeR*::Km(pSYI) (◆), respectively, while β-galactosidase activities are represented as bars [KHW(pSYI), black bars; KHWΔ*bpeAB*(pSYI), gray bars; and KHW*bpeR*::Km(pSYI), striped bars]. All measurements were done in triplicate; the means and standard errors are shown.

in the *bpeR*-overexpressing strains, KHW(pUCP*bpeR*) and KHW*bpeR*::Km(pUCP28T*bpeR*), demonstrates that quorum sensing can be inhibited by overexpressing *bpeR* (Fig. 5A, panels V and VI, respectively). Similar results were obtained using

the *A. tumefaciens* NTI indicator strain, except that in this case the autoinducers were detected by the appearance of a blue color on the reporter strain streaked on agar overlaid with X-Gal (data not shown).

TABLE 3. Effect of *bpeAB* and *bpeR* mutations and exogenous C8HSL (100 nM) on invasion of A549 and THP-1 cells by *B. pseudomallei*

| Strain | Inoculum (CFU) | Mean no. of cells recovered at 4 h[a] | | | |
|---|---|---|---|---|---|
| | | A549 cells | | THP-1 cells | |
| | | −C8HSL | +C8HSL | −C8HSL | +C8HSL |
| KHW | $1.5 \times 10^7$ | $7.8 \times 10^4 \pm 1.4 \times 10^4$ | $8.4 \times 10^4 \pm 2.0 \times 10^4$ | $8.5 \times 10^4 \pm 1.7 \times 10^4$ | $6.4 \times 10^4 \pm 7.2 \times 10^3$ |
| KHWΔ*bpeAB* | $1.4 \times 10^7$ | $2.6 \times 10^2 \pm 2.3 \times 10^2$ | $7.1 \times 10^4 \pm 2.1 \times 10^3$ | $6.6 \times 10^2 \pm 1.0 \times 10^2$ | $6.8 \times 10^4 \pm 5.3 \times 10^3$ |
| KHWΔ*bpeAB*(pUCP28T*bpeAB*) | $1.5 \times 10^7$ | $7.2 \times 10^4 \pm 2.2 \times 10^4$ | $8.9 \times 10^4 \pm 1.5 \times 10^4$ | $9.0 \times 10^4 \pm 1.2 \times 10^4$ | $8.1 \times 10^4 \pm 1.5 \times 10^4$ |
| KHW*bpeR*::Km | $1.7 \times 10^7$ | $4.3 \times 10^4 \pm 9 \times 10^3$ | $8.3 \times 10^4 \pm 1.5 \times 10^4$ | $7.6 \times 10^4 \pm 2.0 \times 10^4$ | $7.3 \times 10^4 \pm 1.6 \times 10^4$ |
| KHW*bpeR*::Km(pUCP28T*bpeR*) | $1.8 \times 10^7$ | $28 \pm 17$ | $1.1 \times 10^5 \pm 1.3 \times 10^4$ | $100 \pm 15$ | $8.3 \times 10^4 \pm 1.3 \times 10^4$ |
| KHW(pUCP28T*bpeR*) | $1.6 \times 10^7$ | $35 \pm 24$ | $9.0 \times 10^4 \pm 6.6 \times 10^3$ | $87 \pm 14$ | $7.5 \times 10^4 \pm 1.4 \times 10^4$ |

[a] Values are means ± standard deviations.

**BpeAB-OprB function affects growth-phase-dependent expression of the autoinducer synthase BpsI.** Since a reduction in autoinducer production in KHWΔ*bpeAB* may be the result of either the absence of autoinducer efflux or repression of autoinducer synthase gene expression, we determined the expression of *bpsI*, which encodes an autoinducer synthase, in KHW and its isogenic mutants (34). It was recently reported that *B. pseudomallei* has three *luxIR* homologs which produce a variety of acyl-HSLs, including C8HSL, C10HSL, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy-C8HSL), *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-hydroxy-C10HSL), and *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14HSL) (37). BpsI synthesizes mainly C8HSL (34).

Wild-type *B. pseudomallei* KHW entered stationary phase at 15 h after culture in AB medium containing 20 mM glycerol and 0.2% (wt/vol) Casamino Acids (Fig. 5B). In comparison, the doubling time in KHWΔ*bpeAB* was almost twice as long and stationary phase was attained only after 27 h of culture. We introduced the *bpsI*$_{promoter}$-*lacZ* plasmid, pSYI, into KHW and KHWΔ*bpeAB* to study the effect of BpeAB-OprB on *bpsI* expression. In the *bpeAB* mutant, *bpsI*$_{promoter-lacZ}$ expression remained low for the duration of culture, finally attaining about half the level of that in KHW. In KHW*bpeR*::Km, which had functional BpeAB-OprB, the growth properties and levels of *bpsI*$_{promoter-lacZ}$ expression after 30 h of culture were similar to those in KHW, which verifies that the absence of growth-phase-dependent expression of *bpsI* in the *bpeAB* mutant was indeed a consequence of the defective efflux pump (Fig. 5B). We have also cultured the *bpeAB*-null mutant under conditions similar to those described in Fig. 5B, but in the presence of 100 nM C8HSL, and observed that the growth of the *bpeAB*-null mutant was not restored to wild-type level (data not shown). The inclusion of 100 nM C8HSL in the culture medium also

did not restore growth-phase-dependent *bpsI*$_{promoter}$-*lacZ* expression in KHWΔ*bpeAB*, suggesting that intracellular concentrations of C8HSL might be high, possibly due to impaired efflux, and the accumulation of C8HSL in the *bpeAB* mutant might have resulted in a negative-feedback effect on *bpsI* expression.

**Role of BpeAB-OprB in invasion of epithelial and macrophage cell lines.** Since any effect on quorum sensing could potentially affect virulence, we next determined if BpeAB-OprB would have an effect on the invasion of human lung epithelial (A549) and macrophage (THP-1) cells by *B. pseudomallei*. In the absence of BpeAB-OprB function, the invasion of both A549 and THP-1 cells by KHWΔ*bpeAB* as well as the *bpeR*-overexpressing strains KHW*bpeR*::Km(pUCP28T*bpeR*) and KHW(pUCP28T*bpeR*) was significantly attenuated (Table 3). Interestingly, exogenous addition of 100 nM C8HSL to the A549 and THP-1 cell culture media could restore to wild-type levels this defect in cell invasion in the strains KHWΔ*bpeAB*, KHW*bpeR*::Km(pUCP28T*bpeR*), and KHW(pUCP28T*bpeR*). Taken together, these results demonstrate that (i) the BpeAB-OprB efflux function is essential for cell invasion by *B. pseduomallei* and (ii) the reduced invasiveness of KHWΔ*bpeAB* was probably due to its impaired quorum-sensing mechanism. Additionally, our data also demonstrate that it was possible to attenuate *B. pseudomallei* virulence by overexpressing the BpeR repressor. It is interesting that KHW, which overexpressed the BpeR repressor, was approximately 10-fold more attenuated in cell invasion of both cell lines than was the *bpeAB*-null mutant (Table 3). However, upon the addition of exogenous C8HSL, the invasiveness of the *bpeR*-overexpressing strain and that of the *bpeAB*-null mutants were equally restored to wild-type levels.

**Role of BpeAB-OprB in cytotoxicity of *B. pseudomallei*.** The cytotoxic effects of both KHWΔ*bpeAB* and the *bpeR*-over-

TABLE 4. Effect of *bpeAB* and *bpeR* mutations and exogenous C8HSL (100 nM) on cytotoxicity of *B. pseudomallei*[a]

| Strain | Inoculum (CFU) | Cytotoxicity (%) in cells: | | | |
|---|---|---|---|---|---|
| | | A549 | | THP-1 | |
| | | −C8HSL | +C8HSL | −C8HSL | +C8HSL |
| KHW | $1.5 \times 10^7$ | $43.9 \pm 3.0$ | $48.7 \pm 1.9$ | $41.6 \pm 0.6$ | $57.1 \pm 0.7$ |
| KHWΔ*bpeAB* | $1.4 \times 10^7$ | $2.8 \pm 1.1$ | $21.7 \pm 4.7$ | $0 \pm 0.6$ | $30.7 \pm 3.7$ |
| KHWΔ*bpeAB*(pUCP28T*bpeAB*) | $1.5 \times 10^7$ | $50.1 \pm 4.9$ | $50.6 \pm 2.6$ | $21.2 \pm 1.4$ | $55.7 \pm 0.7$ |
| KHW*bpeR*::Km | $1.7 \times 10^7$ | $52.1 \pm 3.8$ | $48.8 \pm 0.9$ | $41.9 \pm 0.7$ | $55.3 \pm 0.8$ |
| KHW*bpeR*::Km(pUCP28T*bpeR*) | $1.8 \times 10^7$ | $11.0 \pm 3.2$ | $13.7 \pm 3.8$ | $0.6 \pm 1.0$ | $27.1 \pm 0.5$ |
| KHW(pUCP28T*bpeR*) | $1.6 \times 10^7$ | $6.2 \pm 6.4$ | $9.0 \pm 1.6$ | $0 \pm 0.7$ | $27.4 \pm 0.5$ |

[a] Values are means ± standard deviations.
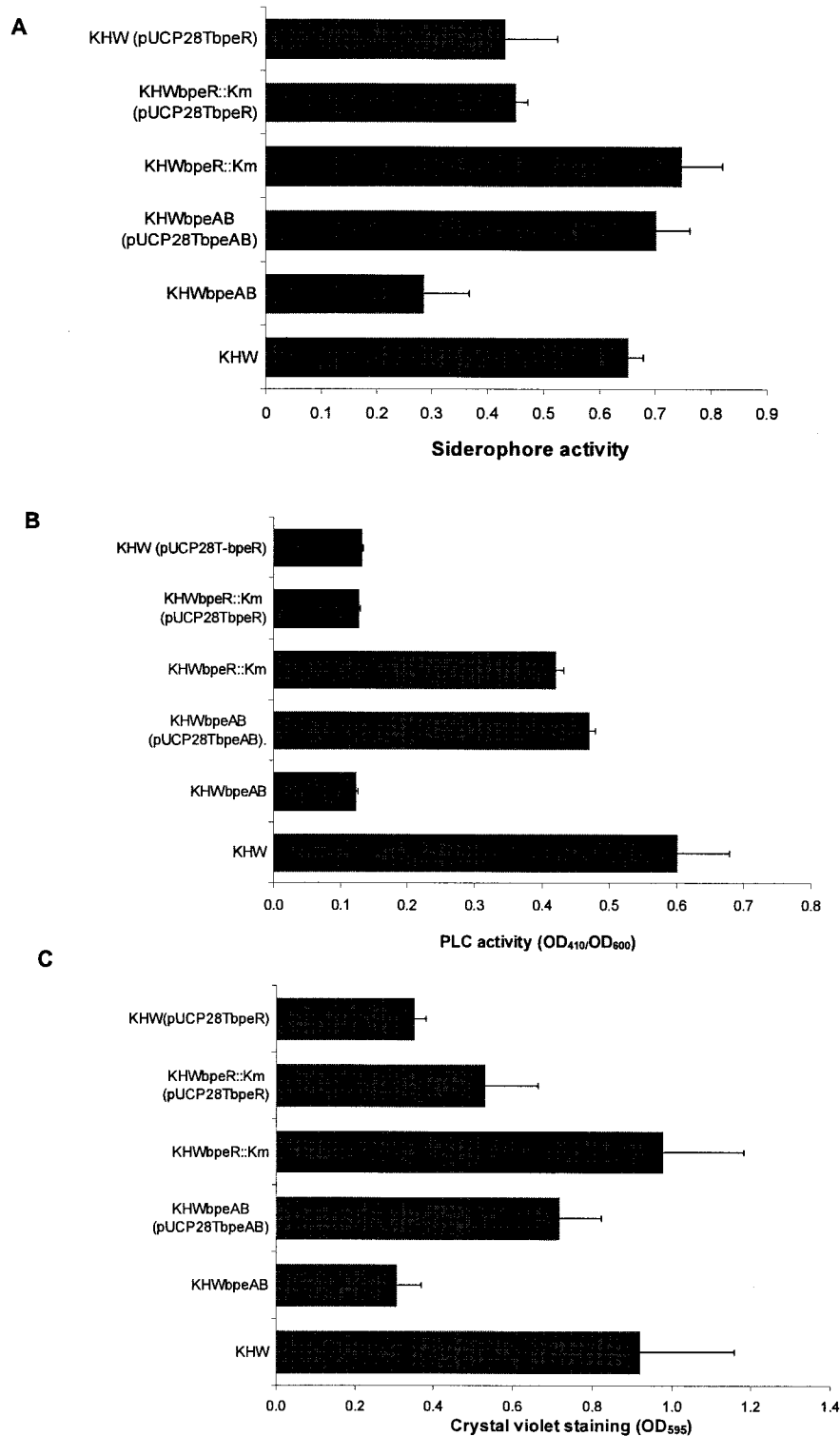
FIG. 6. Effect of BpeAB-OprB on siderophore and phospholipase C production and biofilm formation in *B. pseudomallei*. Siderophore activities, phospholipase C activities, and biofilm formation were measured in KHW, KHWΔ*bpeAB*, KHWΔ*bpeAB* complemented with pUCP28T*bpeAB*, KHW*bpeR*::Km, KHW*bpeR*::Km complemented with pUCP28T*bpeR*, and KHW harboring pUCP28T*bpeR*, respectively. (A) Optimal siderophore production in *B. pseudomallei* is dependent on the BpeAB-OprB function, and overexpression of *bpeR* in KHW*bpeR*::Km(pUCP28T*bpeR*) and KHW(pUCP28T*bpeR*) reduced siderophore production. Siderophore activities were assayed in the supernatants of 24-h-old cultures and were determined by measuring the differential in $OD_{630}$ readings between the test and the sample blank. The values shown have been normalized for cell density by being expressed as a ratio of $\Delta OD_{630}/OD_{600}$. (B) Optimal PLC secretion by *B. pseudomallei* KHW is dependent on BpeAB-OprB, and overexpression of *bpeR* in KHW*bpeR*::Km(pUCP28T*bpeR*) and KHW(pUCP28T*bpeR*) inhibited PLC secretion. PLC activities were determined in the supernatants of 24-h cultures, and the values shown have been normalized for cell density by being expressed as a ratio of $OD_{410}/OD_{600}$. (C) Optimal biofilm formation is also dependent on BpeAB-OprB. Biofilm formation was significantly reduced in KHWΔ*bpeAB* and KHW overexpressing *bpeR*. Each bar is the average reading (± standard deviation) from three independent experiments.

expressing strains, KHW*bpeR*::Km(pUCP28T-*bpeR*) and KHW(pUCP28T-*bpeR*), on both A549 and THP-1 were significantly reduced (Table 4). Forty-four percent of A549 cells and 42% of THP-1 cells were killed after exposure to KHW for 4 h. Compared to KHW, the cytotoxicity of KHWΔ*bpeAB*, KHW*bpeR*::Km(pUCP28T*bpeR*), and KHW(pUCP28T*bpeR*) to A549 was significantly attenuated to 3%, 11%, and 6%, respectively, while their cytotoxicity to THP-1 cells was completely attenuated (Table 4). Addition of exogenous C8HSL could only partially restore the cytotoxicity of KHWΔ*bpeAB*, KHW*bpeR*::Km(pUCP28T*bpeR*), and KHW(pUCP28T*bpeR*) to both types of cells.

A difference between the two cell types is that the cytotoxicity towards A549 cells was fully restored to wild-type level when KHWΔ*bpeAB* was complemented in *trans* with pUCP28T*bpeAB*, as opposed to its cytotoxicity towards THP-1 cells, which could be only partially restored to 50% of wild-type level in the complemented mutant. The *bpeR* mutation had no effect on the cytotoxicity of KHW to both types of cells. These data showed that the BpeAB-OprB function is also required for the cytotoxicity of *B. pseudomallei* towards A549 and THP-1 cells and that overexpression of the BpeR repressor could attenuate cytotoxicity. The attenuated virulence of the strains defective in *bpeAB-oprB* expression correlates well with their lack of autoinducer production, suggesting the importance of quorum sensing in the cytotoxic effect of *B. pseudomallei*.

**BpeAB-OprB function is required for optimal biofilm formation and for the production of siderophores and phospholipase C.** We have previously shown that the *B. pseudomallei* BpsIR quorum-sensing system regulates siderophore and phospholipase C production (34). We have also observed a reduction in biofilm formation in the *bpsI* and *bpsR* mutants (data not shown). Since BpeAB-OprB affects quorum sensing in *B. pseudomallei*, we were interested to know if these quorum-sensing-controlled cellular processes would also be affected by BpeAB-OprB. Siderophore production was reduced by 50% in the KHWΔ*bpeAB* mutant but was reduced by only 30% in the strains overexpressing *bpeR* (Fig. 6A). The reduction of PLC production in the *bpeAB* mutant and the strains overexpressing *bpeR* was more significant. PLC activity detected in KHWΔ*bpeAB*, KHW*bpeR*::Km(pUCP28T*bpeR*), and KHW(pUCP28T*bpeR*) was only about 20% of the wild-type KHW level (Fig. 6B). Biofilm formation was also significantly reduced in the *bpeAB* mutant and the *bpeR*-overexpressing strains. In KHWΔ*bpeAB*, KHW*bpeR*::Km(pUCP28T*bpeR*), and KHW(pUCP28T*bpeR*), biofilm formation was 33%, 58%, and 38% of that in KHW, respectively (Fig. 6C). These data showed that quorum-sensing-controlled processes like optimal siderophore and phospholipase production, as well as biofilm formation, are dependent on BpeAB-OprB function. Overexpression of the BpeR repressor could also partially inhibit these processes.

## DISCUSSION

The *B. pseudomallei* BpeAB-OprB efflux system is inducible by its substrate erythromycin. Since subinhibitory concentrations of erythromycin have been reported to induce the expression of *luxI* and *lasI* promoters and thus trigger the expression

of many quorum-controlled target genes (14), it was necessary to distinguish whether *bpeAB* expression was induced by erythromycin because of its interaction with the BpeR repressor or if it was due to a more general transcriptional modulation of gene expression by subinhibitory concentrations of wide-ranging antibiotics as described by Goh et al. (14). We addressed this issue by using a subinhibitory concentration (0.1× MIC) of amoxicillin, an antibiotic which is not a substrate of BpeAB-OprB, to study its effect on *bpeAB*$_{promoter-lacZ}$ expression in KHW. Unlike erythromycin, subinhibitory concentrations of amoxicillin did not induce *bpeAB*$_{promoter}$-*lacZ* and *bpeR*$_{promoter}$-*lacZ* expression in the wild-type background (data not shown). Thus, the induction of *bpeAB* expression by low-dose erythromycin appears to be a specific effect of erythromycin on *bpeAB* expression. Additionally, instead of using subinhibitory concentrations of erythromycin, we also performed transient assays by exposing the KHW(pCYY*bpeAB*) cultures to high doses of erythromycin at 5× MIC and 10× MIC for 30 min instead of 4 h. At both doses of erythromycin tested, *bpeAB* expression remained inducible in a dose-dependent manner, also confirming that the induction of *bpeAB* expression by erythromycin was a substrate-specific event (data not shown).

The low basal *bpeAB-oprB* expression present in wild-type cells may be attributed to a titration of the BpeR repressor by multiple copies of the *bpeAB*$_{promoter}$-*lacZ* fusion plasmid used in this study (Fig. 2A). KHW*bpeR*::Km, which lacks the BpeR repressor, showed a basal *bpeAB-oprB* expression which was about 3.5-fold higher than that of the wild type. This consequently resulted in its relatively higher MICs and MBCs for gentamicin, streptomycin, and erythromycin (Table 2). These data are consistent with the role of BpeR as a repressor of *bpeAB-oprB* expression. We have also observed a delay in the erythromycin-induced expression of *bpeR*$_{promoter-lacZ}$ expression in wild-type cells, compared to that of *bpeAB*$_{promoter-lacZ}$ expression (data not shown). This supports the view that the BpeR repressor interacts with its ligand to regulate *bpeAB-oprB* expression as well as its own expression. However, it was unexpected that the *bpeAB-oprB* expression in KHW*bpeR*::Km would remain inducible by erythromycin to levels comparable to that of the wild-type parental strain. An explanation would be the participation of another transcriptional regulator which recognizes erythromycin as a ligand and binds to a different site in the *bpeAB-oprB* regulatory region. Alternatively, a repressor protein of another RND pump (e.g., AmrR) which uses erythromycin as a ligand could share the *bpeAB-oprB* regulatory region. It has been reported that two promoters transcribe the *P. aeruginosa* *mexAB-oprM* operon—one of them regulated by the MexR repressor but the other not (11).

We showed that the expression of *bpeAB-oprB* is growth phase dependent and that that of *bpeR* is inducible upon entry into stationary phase. This could be explained if *bpeAB-oprB* expression is either (i) induced by a metabolite which is a substrate of the pump and accumulates with cell density or (ii) activated by a transcriptional regulator which controls the expression of genes at stationary phase or (iii) regulated by quorum sensing. *mexAB-oprM* expression in *P. aeruginosa* is similarly growth phase dependent, and the regulation of its growth-phase-dependent expression does not involve the MexR repressor (13). Our data have shown that *bpeAB-oprB*

expression could be induced by exogenous autoinducers. BpeAB-OprB could be involved in the efflux of autoinducers, and the impairment of this efflux in the *bpeAB*-null mutant or the *bpeR*-overexpressing strains would result in an accumulation of autoinducers which would then exert a negative feedback on the expression of the autoinducer synthase. This would explain the attenuated virulence phenotype of the *bpeAB*-null mutant and the *bpeR*-overexpressing strains. Three pairs of *luxIR* homologs and two other *luxR* homologs have been identified in *B. pseudomallei*. Together they are responsible for the production of several acyl-homoserine lactone autoinducers: C8HSL, C10HSL, 3-hydroxy-C8HSL, 3-hydroxy-C10HSL, and 3-oxo-C14HSL (34, 37, 38). Using early-exponential-phase cultures when *bpeAB-oprB* expression was low, we showed that the induction of *bpeAB-oprB* expression could be advanced to the exponential phase by the exogenous addition of 100 nM C8HSL or C10HSL to the culture medium. It is noted that the response of *bpeAB-oprB* expression to such high concentrations of exogenous C8HSL or C10HSL might not necessarily imply that *bpeAB-oprB* expression is directly regulated by quorum sensing, as the acyl-HSLs could have activated a stationary-phase transcriptional regulator. The *P. aeruginosa* RpoS is an alternative sigma factor responsible for the switching-on of gene expression at stationary phase and is involved in the expression of 40% of quorum-controlled genes (33). *rpoS* expression is activated by quorum sensing, and among target genes regulated by both quorum sensing and RpoS there are probable RND efflux transporters (19, 33). Since the expression of *rpoS* in *B. pseudomallei* is also growth phase dependent (35), it is therefore difficult to distinguish if the induction of *bpeAB-oprB* expression at stationary phase is due to activation by RpoS or quorum sensing or both.

Although we observed that *bpeAB* expression could be activated by exogenous autoinducers, it was more responsive to C8HSL than to C10HSL, and only at high concentrations of C8HSL (>80 nM). Likewise, it was recently reported that the expression of *P. aeruginosa mexAB-oprM* was differentially enhanced by the exogenous addition of C4HSL and 3-oxo-C12HSL (20), although its expression was not regulated by the LasIR quorum-sensing system (13). We have also identified a *las/lux* box motif in the intergenic region between *bpeR* and *bpeAB-oprB*, but the significance of this has not been studied. It could perhaps be addressed by studying the effect of exogenous C8HSL and C10HSL on *bpeAB-oprB* expression in a quorum-sensing-null genetic background, even though such a mutant could be difficult to construct because of the presence of multiple *luxIR* homologs in *B. pseudomallei* (37).

Efflux pumps have also been implicated in the efflux of autoinducers, but direct evidence which demonstrates that autoinducers are indeed substrates of efflux pumps is lacking. It is reported that hyperexpression of *P. aeruginosa* MexAB-OprM resulted in a decline in *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C12HSL) levels but had no effect on C4HSL levels (12). Consequentially, it is believed that, although C4HSL freely diffuses into and out of *P. aeruginosa* cells, movement of 3-oxo-C12HSL out of the cells requires active efflux (26). Our data showed that autoinducer production is significantly reduced when BpeAB-OprB function is impaired (Fig. 5A, panels II, V, and VI), and this reduction in autoinducer production seemed to be at the level of autoinducer synthesis,

as the expression of the autoinducer synthase was impaired in the *bpeAB* mutant (Fig. 5B). It is interesting that in KHW*bpeR*::Km, where *bpeAB* expression is up-regulated, *bpsI* expression remained low up to early stationary phase (~18 h) and then increased to wild-type levels thereafter. This was in contrast to *bpsI* expression in the KHWΔ*bpeAB* mutant, which was not induced in stationary phase. This again points to other factors besides BpeR being involved in the regulation of *bpeAB-oprB* expression. We have also measured *bpsI* expression in the *bpeAB*-null mutant and found that *bpsI* expression was not inducible when cells were cultured up to 6 h in the presence of 100 nM exogenous C8HSL (data not shown). This suggests that C8HSL might be effluxed by BpeAB-OprB and that its high intracellular concentration in the *bpeAB* mutant could have a negative feedback inhibitory effect on the *bpsI* expression. Any inhibition of autoinducer synthase expression would therefore have a negative effect on quorum-regulated gene expression.

Growth impairment was observed in the KHWΔ*bpeAB* mutant compared to KHW, both when cultured in AB medium supplemented with glycerol and Casamino Acids and when cultured in LB medium. The KHWΔ*bpeAB* mutant had a longer doubling time and attained stationary phase only after >80 h although microscopic examination of the negatively stained cells by transmission electron microscopy showed size and length similar to those of KHW cells (data not shown). The failure to restore the growth impairment of the *bpeAB*-null mutant to wild-type levels in the presence of 100 nM C8HSL suggests that the cause of the growth defect of the *bpeAB*-null mutant is probably not regulated by C8HSL. It is also plausible that there is an accumulation in the KHWΔ*bpeAB* mutant of a metabolite which is a physiological substrate of the BpeAB-OprB efflux pump and which has an impact on cell division. Polyamines, whose biosynthesis shares the same pathway as that of acyl-homoserine lactones, are plausible candidates (25, 36). The intracellular level of polyamines, which is tightly regulated, is an important control of cell division in *E. coli* (16).

Importantly, the impairment of the BpeAB-OprB pump either by the *bpeAB*-null mutation or by overexpression of *bpeR* had a dramatic effect on virulence attenuation of *B. pseudomallei*. These strains showed significant impairment in cell invasion of human lung epithelial (A549) and macrophage (THP-1) cells (Table 3). The cytotoxic effect of these strains on A549 and THP-1 was also significantly attenuated (Table 4). The partial restoration of cytotoxicity of the KHWΔ*bpeAB* mutant and the *bpeR*-overexpressing strains, KHW*bpeR*::Km(pUCP28T*bpeR*) and KHW(pUCP28T*bpeR*), by the addition of exogenous C8HSL supports the notion that the BpeAB-OprB efflux function is important for optimal autoinducer synthesis. It is possible that the addition of exogenous C8HSL, which failed to induce *bpsI*$_{promoter-lacZ}$ expression in the *bpeAB* mutant, may have a positive effect on the expression of the other *luxIR* homologs in the efflux-impaired *B. pseudomallei*. Such an effect could have restored the invasiveness of these efflux-impaired strains. It remains unclear why the addition of exogenous C8HSL autoinducer to the culture medium was able to partially restore the cytotoxic effect of the *bpeAB* mutant and *bpeR*-overexpressing strains towards THP-1 cells but had no effect on the cytotoxic effect of the same strains towards A549 cells.

It is also interesting that, in the identification of new regulators that modulate quorum sensing in *P. aeruginosa*, a mutation in a probable RND-like efflux transporter was found to significantly down-regulate the quorum-sensing-dependent *lecA*::*lux* expression (9). Our study has shown that the BpeAB-OprB function is important for optimal production of virulence factors such as siderophore and phospholipase C and for biofilm formation. The BpeAB-OprB function is thus an important virulence determinant of *B. pseudomallei*.

Future work will include the identification of the physiological substrate of the BpeAB-OprB efflux pump and the BpeR-dependent and BpeR-independent regulatory sites in the *bpeA-bpeR* intergenic region. Intermediary metabolites in the autoinducer biosynthetic pathway will be examined. Polyamines, such as spermidine and putrescine, which are important for many cellular functions including cell division, are plausible candidates.

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