**Burkholderia pseudomallei** resistance to antibiotics in biofilm-induced conditions is related to efflux pumps

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**INTRODUCTION**

*Burkholderia pseudomallei*, the causative agent of melioidosis, is a non-spore-forming Gram-negative saprophytic bacterium. *B. pseudomallei* can survive in toxic environments and is resistant to various antibiotics and disinfectants used in household processes and hospitals. *B. pseudomallei* has intrinsic resistance to several antibiotics, and several resistance mechanisms have been reported. Loss of penicillin-binding protein 3 in *B. pseudomallei* leads to ceftazidime (CTZ) resistance (Chantaritita et al., 2011). There are several acquired mutations that can lead to CTZ resistance, for example, mutation of β-lactamase gene penA (BPSS0946) such as in penA p167s (Sarovich et al., 2012a; Tribuddharat et al., 2003), penA C69Y (Sam et al., 2009; Sarovich et al., 2012b) and penA –21G upstream region (Sarovich et al., 2012b). *B. pseudomallei* can be grown as microcolonies and biofilm. The biofilm production ability varied among strains but not related to their virulence (Taweechaisupapong et al., 2005). Biofilm formation and lipopolysaccharides of *B. pseudomallei* were found to be related to relapse of melioidosis cases (Limmathurosatskul et al., 2014). Interestingly, biofilms are one of the important candidate mechanisms for antibiotic resistance in various bacteria including *B. pseudomallei*. The biofilm cells of *B. pseudomallei*...
*pseudomallei* played a role as a diffusion barrier for antibiotics that led to a decrease in antibiotic concentrations at their targets (Anderl et al., 2000; Pibalpakdee et al., 2012). Decreased growth and metabolic rate that were due to a limitation of oxygen and nutrients in the development of biofilm cells and persister cells were identified as important factors for antibiotic tolerance in biofilms (Chen, 2011; Nierman et al., 2015; Walters et al., 2003). Persisters in biofilms may exhibit tolerance, which means that cells do not grow in the presence of antibiotics but do not die either. This ability to avoid being killed is the key feature of persisters. Persisters, however, do not form in response to antibiotic treatment, since early exponential cells challenged with antibiotics produced no persisters (Keren et al., 2004). Sawasdiloln et al. (2010) found that growing various strains of *B. pseudomallei* in biofilm formation conditions could induce the bacteria to be resistant to antibiotics commonly used for melioidosis treatment regimens. Biofilm cells of *B. pseudomallei* developed more than 10 times the MIC values when compared to the MIC of its planktonic growth, but cells that were shed from the biofilm layer were still susceptible to tested antibiotics, and all tested strains had the same MIC values when compared to their MIC values in planktonic form. There are several possible mechanisms that make those bacteria resistant in planktonic form such as the up-regulation of intrinsic drug resistance genes or efflux pumps (Biot et al., 2013; Podnecky et al., 2015; Rajendra et al., 2010), but in the case of biofilms, the related resistance in *B. pseudomallei* is still unclear.

Bacterial efflux pump systems are other candidates for antibiotic resistance. Various multidrug-resistant bacteria such as *Escherichia coli* extrude antibiotics through resistance–modulation–division (RND) efflux systems. Multiple antibiotic resistance in *E. coli* includes efflux pump AcrAB to extrude various antibiotics (Okusa et al., 1996). *Burkholderia cenocepacia* J2315 has an RND efflux system which is involved in resistance to some antibiotics and inhibitory substances (Bazzini et al., 2011; Buroni et al., 2009). *Pseudomonas aeruginosa* PA14 growing in the biofilm stage is up-regulated by PA1874–PA1877 encoding genes. This operon has been shown to overexpress only in the biofilm formation process but was expressed less in planktonic cells. These genes had important roles in tobramycin, gentamycin and ciprofloxacin resistance by extrusion activity to reduce antibiotic accumulation in bacterial cells. Mutant strains with these PA1874–PA1877 operons deleted were susceptible to these antibiotics but their ability to form biofilms (Zhang & Mah, 2008) was not affected. *B. pseudomallei* contains at least 10 operons of RND efflux pump components. Seven of them are located on chromosome 1, and another three are on chromosome 2 (Podnecky et al., 2015). The well-characterized RND efflux pumps that are involved in resistance to antibiotics have been well identified, including AmrAB-OpRA, BpeAB-OpRB and BpeEF-OpRC (Kumar et al., 2008; Mima & Schweizer, 2010; Podin et al., 2014; Podnecky et al., 2013).

The mechanisms of resistance of *B. pseudomallei* when growing in biofilm-induced conditions are still incompletely understood. Because *B. pseudomallei* and *P. aeruginosa* are related species (Yabuuchi et al., 1992), then in using *P. aeruginosa* PA14 as a model, the orthologues of PA1874–PA1877 genes in *B. pseudomallei* were defined by using bioinformatics tools. Expression of orthologues and selected efflux pump encoding genes on chromosome 1 during biofilm formation were investigated. To confirm this finding, the universal efflux pump inhibitor (phenylalanine arginine β-naphthylamide, PAβN) was used. PAβN is a short synthetic peptide that was discovered and reported as a competitive-type efflux pump inhibitor (Renau et al., 1999). It has been reported to block the excretion of several aminoglycosides and β-lactam-type antibiotics in doxycycline (DOX)-selected strains of *Burkholderia thailandensis* (Biot et al., 2013). In this study, PAβN was used to inhibit efflux pump function and monitor susceptibility of *B. pseudomallei* biofilm cells. Correlation of efflux transporters and antibiotic resistance during biofilm-inducing conditions was explored.

### METHODS

**Bacterial strains and culture conditions.** *B. pseudomallei* K96243, 1026b, G207, U2704, H777, M10 (biofilm-defective mutant of H777) and FliC knockout mutant MM35 together with *E. coli* ATCC 25922 were used in this study (Table 1). They were grown on Luria–Bertani (LB) agar plates. Mutant strains M10 and MM35 were grown on LB agar plates supplemented with 15 µg ml\(^{-1}\) tetracycline. Biofilms of *B. pseudomallei* were formed in modified Vogel and Bonner’s medium (MVBM) (Taweewchasupapong et al., 2005).

**Bioinformatics analysis of the *P. aeruginosa* efflux transporter orthologous genes in *B. pseudomallei* genomes.** *B. pseudomallei* K96243 orthologous genes of PA1874–PA1877 (GenBank accession nos NP_250565.1, NP_250566.1, NP_250567.1 and NP_250568.1) in the *P. aeruginosa* PA01 genome were identified in the KEGG database and confirmed by reciprocal best search hits (BLASTX) (Moreno-Hagelsieb & Latimer, 2008). The reciprocal best search hits were BPS1661, BPSL1664 and BPSL1665 (GenBank accession nos YP_108272.1, YP_108275.1 and YP_108276.1). They were identified as orthologous genes on *B. pseudomallei* K96243 chromosome 1. Subcellular localizations were predicted by PSORTb, and protein domains were compared to confirm the characters of orthologous genes (Yu et al., 2010). The results of orthologous gene confirmation and sub-localization predictions are shown in the supplementary material (Tables S1–S4, available in the online Supplementary Material).

**Detection of putative efflux transporter genes on *B. pseudomallei* chromosomes by PCR.** Specific primers of BPS1661, BPSL1664 and BPSL1665, the orthologues of PA1874–PA1877 genes with encoding genes for well-known RND efflux transporters and the bpeA gene of BpeAB-OpRA were designed by using Primer-BLAST (Ye et al., 2012). Primers for bpeB, bpeR and amrB, coding for the RND efflux transporter located on chromosome 1, were obtained (Kumar et al., 2008; Mima & Schweizer, 2010). Their sequences, properties and amplified product sizes are shown in Table 2. Regions for the amplification target of each gene are shown in Fig. 1. Synthesized primers were used for amplification of fragments of candidate genes from various *B. pseudomallei* strains including K96243, 1026b, G207, U2704, H777, M10 and MM35 by conventional PCR with a Veriti 96-Well Thermal Cycler (Applied Biosystems). Briefly, PCR mixture (RBC Bioscience) with genomic DNA templates was heated to 94 °C for 10 min and then amplified for 35 cycles by denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. Then the amplification process for the final extension was done at 72 °C for 7 min. When amplification was completed, amplicons were visualized using agarose gel electrophoresis.
Detection of efflux transporter encoding genes in the *B. pseudomallei* biofilms. The biofilms of *B. pseudomallei* K96243 were induced as described by Sawasdidoln et al. (2010). Briefly, overnight cultures of *B. pseudomallei* K96243 in MVBM were adjusted to $10^7$ c.f.u. ml$^{-1}$, and then 500 µl of bacterial suspension was inoculated into each well of flat-bottom 48-well plates. The inoculated plates were incubated by shaking at 100 r.p.m. at 37°C for 3 h for early attached biofilm cells and for 24 and 48 h for biofilm cells and were then washed with PBS (pH 7.2) to remove unattached cells, and the biofilm cells were harvested by scraping with sterile pipette tips. At this point, we hoped to have found significantly different gene expressions when compared to previous time points. The total RNAs were isolated by TRIzol reagent (Invitrogen) as recommended by the manufacturer. Complementary DNAs of planktonic cells and biofilm cells were synthesized by reverse transcriptase PCR (RT-PCR) (Invitrogen) and then used for determination of expression levels of genes by real-time RT-PCR with the Light Cycler 480 platform (Roche). All relative expression levels were calculated by the ΔΔCt method and normalized with 16S rRNA gene (Chirakul et al., 2014).

Real-time RT-PCR. The complementary DNA of mid-log planktonic cells and 3 h, 1-day and 2-day biofilms of *B. pseudomallei* was used to investigate relative quantification of BPSL1661, BPSL1664 and BPSL1665 efflux pump candidate genes and amrR, bpeA and bpeB, encoding genes for components of AmrAB-OprA and BpeAB-OprB, the RND efflux pump systems on chromosome 1; bpeR, the regulator protein encoding
gene of the BpeAB-OprB efflux pump system; and the housekeeping gene, 16S rRNA. All of them were amplified with primers that are the same primers used in conventional PCR (Table 2), as each product gave only one peak when confirmed by endpoint and melting peak analysis. Reactions were amplified by LightCycler SYBR Green I Master (Roche) under the following conditions: held at 95°C for 5 min, followed by amplification at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. SYBR Green fluorescence signals were captured by acquisition at 72°C for each cycle of 45 cycles followed by a melting curve analysis and cooling. Amplifications were controlled, and data were analysed by LC480 LightCycler software version 1.5.1 from Roche Diagnostics. PCRs were finally amplified at 95°C.

**Susceptibility of planktonic cells to antibiotics.** Susceptibility of planktonic cells to antibiotics with or without a universal efflux pump inhibitor was determined by the standard broth microdilution assay. Briefly, overnight cultures of *B. pseudomallei* K96243 were adjusted to cell densities of 10^5 c.f.u. ml^-1, then challenged with two-fold serial dilutions of CTZ (1–1024 µg ml^-1), DOX (0.12–256 µg ml^-1), imipenem (IMN) (0.12–256 µg ml^-1) and PAβN (1–1024 µg ml^-1) in Mueller–Hinton (MH) broth and then incubated at 37°C for 24 h. Growth was measured by observations of optical density at 620 nm by an ELISA plate reader (Sunrise; Tecan). OD_{620}<0.1 was evidence of inhibited growth. The lowest concentrations of antibiotics that could inhibit growth of bacterial cells were reported as the MICs.

To determine susceptibility of biofilms to antibiotics and the universal efflux pump inhibitors, *B. pseudomallei* K96243 biofilms were prepared in MVBM broth with the Calgary Biofilm Device (CBD) as previously described by Sawasdidoln et al. (2010). The 1-day biofilms were challenged with two-fold serial dilutions of 1 to 1024 µg ml^-1 CTZ, 0.12 to 256 µg ml^-1 DOX, 0.12 to 256 µg ml^-1 IMN and 1 to 1024 µg ml^-1 PAβN in MH broth for 24 h at 37°C. The challenged plates were replaced with transparent sterile lids, and then the optical densities at 620 nm were measured by the ELISA reader as described above. The challenged biofilms on the CBD lids that were removed from the challenged plates were washed with sterile PBS (pH 7.2) to remove excess antibiotics or inhibitor, and then the lids were placed on the recovery plates which contained fresh MH broth and then incubated at 37°C for 24 h. Growth of recovered biofilms on the CBD lids that were removed from the challenged plates was monitored by observing the optical densities at 620 nm. OD_{620}<0.1 was evidence of inhibitory growth. Media without inoculation served as the negative control. The lowest concentration of antibiotics in

### Table 2. Primer sequences, their properties and tentative products amplified

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<tr>
<th>GenBank accession no.</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference or source</th>
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<td>AY305818.1</td>
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<td>16S rRNA-F</td>
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<td>194</td>
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<td>BPSL1661</td>
<td>Hypothetical protein, putative haemolysin-like protein</td>
<td>BPSL1661-F</td>
<td>GGCATTTACACGA-TGCCAGTC</td>
<td>183</td>
<td>This study</td>
</tr>
<tr>
<td>YP_108275.1</td>
<td>BPSL1664</td>
<td>Hypothetical protein, toxin transport-related membrane protein</td>
<td>BPSL1664-F</td>
<td>GATTTGCAGGGTGCA-GAAA</td>
<td>200</td>
<td>This study</td>
</tr>
<tr>
<td>YP_108276.1</td>
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<td>Hypothetical protein, toxin-related secretion protein</td>
<td>BPSL1665-F</td>
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<td>186</td>
<td>This study</td>
</tr>
<tr>
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<td>126</td>
<td>Kumar et al. (2008)</td>
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<td>195</td>
<td>This study</td>
</tr>
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<td>GCCAAATCCCTGA-TCTGGTG</td>
<td>75</td>
<td>Mima &amp; Schweizer (2010)</td>
</tr>
</tbody>
</table>
the wells with no growth detected from the challenged plates and recovery plates was reported as minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC). All experiments were done in duplicate.

**Inhibition of efflux transporters in B. pseudomallei biofilm.** The 1-day biofilms of B. pseudomallei were prepared in flat-bottom 96-well plates with CBD lids as previously described by Sawasdidoln et al. (2010). The CBD lids were washed and placed on the challenged plates which contained two-fold serial dilutions of antibiotics (1–1024 µg ml⁻¹ CTZ, 0.12–256 µg ml⁻¹ DOX and 0.12–256 µg ml⁻¹ IMN) supplemented with 0, 50 and 100 µg ml⁻¹ PAβN, universal efflux pump inhibitor. MBIC and MBEC values were determined as previously described above and compared.

**Antibiotic accumulation assay.** The 1-day biofilms of B. pseudomallei K96243 in MVBM broth were prepared in 48-well plates; then 24 h later, they were challenged at 37°C with MH broth containing antibiotics at the MIC resistance cut-off concentrations (32 µg ml⁻¹ CTZ, 16 µg ml⁻¹ DOX and 16 µg ml⁻¹ IMN), biofilm sub-eradication concentrations (0.5 MBEC: 512 µg ml⁻¹ CTZ, 64 µg ml⁻¹ DOX and 128 µg ml⁻¹ IMN) and biofilm eradication concentrations (MBEC: 1024 µg ml⁻¹ CTZ, 128 µg ml⁻¹ DOX and 256 µg ml⁻¹ IMN) supplemented with final concentrations of 50 and 100 µg ml⁻¹ PAβN. Culture media were removed and the biofilms were triple washed with sterile water for injection to remove unattached cells and excess bacteria. Lysis buffer (0.1 M glycine, pH 3.0) was added and then the mix shaken at 100 r.p.m. at 37°C for 24 h to allow cell lysis to extract the intracellular accumulated antibiotic. Cell lysates of each treatment were collected to be challenged at 37°C, 0.12–PAβN for 1 h and then significantly decreased in the 1-and 2-day biofilms (Fig. 3b). In contrast, the bpeA encoding gene was found to be expressed significantly lower only at 3 h (Fig. 3e). Although amrB relative expression levels were found at 1.02- and 0.99-fold in 3 h and 1-day biofilms, the expression levels slightly increased to 2.07-fold in 2-day biofilms (Fig. 3d). Interestingly, BPSL1665 was found to be significantly expressed through the biofilm growing stages from 3 h to the 2-day biofilms (Fig. 3c). The expression levels of BPSL1665 were significantly increased from planktonic to 2.8- and 10.74-fold in 1-and 2-day biofilms. These results indicated that BPSL1665, the encoding gene that might be important in growing B. pseudomallei biofilm induction conditions, might be related to resistance to antibiotics.

**Statistical analysis.** The Student’s t-test was performed by using the Statistical Package for Social Science (SPSS) version 14; the P<0.05 threshold was used to assign significance.

**RESULTS**

**BPSL1661, BPSL1664 and BPSL1665 genes in the B. pseudomallei genome are orthologues of P. aeruginosa efflux pumps**

Orthologous genes of PA1874–PA1877 in the B. pseudomallei genome were identified by an orthologue search in the KEGG database. Reciprocal best search hits with the tBLASTx, protein domain and subcellular localization were predicted and compared (Tables S1–S4). The results indicated that BPSL1661, BPSL1664 and BPSL1665 genes are orthologues of these genes. Genomic DNA from B. pseudomallei K96243, 1026b, G207, U2704, H777, M10 and MM35 was used to amplify the coding region of the B. pseudomallei genome for conventional PCR. The designed primers could amplify those genes in all bacterial strains tested (Fig. 2).

**BPSL1665 genes were significantly expressed in the conditions of B. pseudomallei biofilm formation**

Relative expression levels of candidate genes were determined at 3 h (early attachment) and days 1 and 2 of biofilm formation conditions and compared to planktonic cells. The 16S rRNA encoding gene was used as the housekeeping normalization gene. The results showed that the relative expression level of the BPSL1661 encoding gene was decreased significantly at a relative fold change of biofilms of 0.63 for 3 h, 0.52 for 1-day and 0.29 for 2-day biofilms (Fig. 3a). In the case of bpeB and bpeR, the encoding genes for cytoplasmic membrane protein and the repressor of BpeAB-OprC RND efflux transporter system, they gave lower expression in 3 h and were significant in days 1 and 2 biofilms (0.48-, 0.14- and 0.15-fold for bpeB and 0.33-, 0.08- and 0.29-fold for bpeR) (Fig. 3f, g). For BPSL1664, the relative expression levels were similar to the expression in planktonic cells of 0.79-fold at 3 h and then significantly decreased in the 1-and 2-day biofilms (Fig. 3b). For B. pseudomallei K96243 was more resistant to CTZ, DOX and IMN when growing in biofilm-inducing conditions (Fig. 4). The MIC values for planktonic B. pseudomallei K96243 were

![Fig. 2. PCR products of BPSL1661, BPSL1664, BPSL1665, bpeA, bpeB, bpeR and amrB. Specific primers listed in Table 2 were used to amplify the genomic DNA of various genes in different bacterial strains, which was analysed on a 1.8% agarose gel.](image-url)
Fig. 3. Relative expression levels of BPSL1661 (a), BPSL1664 (b), BPSL1665 (c), amrB (d), bpeA (e), bpeB (f) and bpeR (g) of \( B. \) pseudomallei K96243 biofilms compared to planktonic cells and presented as fold changes. The values were determined by real-time RT-PCR, and calculations were done using the \( \Delta \Delta C_t \) method. Data were normalized by using 16S rRNA genes as the housekeeping control. Each bar represents an average relative fold change from duplication; error bars indicate SD. The asterisks indicate significant differences (*\( P < 0.05 \), **\( P < 0.01 \)).
2, 1 and 0.25 µg ml\(^{-1}\) for CTZ, DOX and IMN, respectively (Fig. 5a–c). The results showed that the MBICs were slightly higher than MICs for planktonic cells in the case of CTZ and DOX treatment (four to eight times increased), but in the case of IMN treatment, the MICs and MBICs were found to be similar (only two times increased). The MBEC values of K96243 biofilm, however, were found to be 512, 128 and 1024 times increased when compared to MIC values for planktonic, shedding planktonic and biofilm cells. The dashed lines indicate the MIC cut-off point of each drug.

**DISCUSSION**

*B. pseudomallei* has a high potential for resistance to several kinds of antibiotics when it grows in the biofilm stage. Not only high biofilm production strains but also medium, low and biofilm-deficient mutant strains were able to develop resistance to several antibiotics when they were growing in biofilm-inducing conditions (Sawasdidoln et al., 2010). Those results indicated that the resistance may not be due to the biofilm itself but there might be other factors that are stimulated in the biofilm-growing conditions. Moreover, a previous report revealed that a high biofilm production strain gave lower diffusion of CTZ and trimethoprim/sulfamethoxazole than the biofilm-deficient strain (Pibalpakdee et al., 2012). Not only environmental survival factors but also the critical role of biofilm production in relapse of melioidosis have been reported (Limmathurosakul et al., 2014). In the case of *P. aeruginosa* PA01, the researchers found that the PA1874–PA1877-specific efflux pump encoding genes were expressed only in the biofilm stimulation condition, and these genes played a critical role in antibiotic resistance when *P. aeruginosa* was grown in the biofilm stage (Zhang & Mah, 2008). In this study, it was demonstrated that *P. aeruginosa* orthologous genes of biofilm-specific efflux pumps could also be found in *B. pseudomallei*.

To determine whether the efflux transporter inhibitory effect of universal inhibitors led to an increased susceptibility of biofilm cells to CTZ and DOX by an increased accumulation of intracellular antibiotics, intracellular antibiotics from treated biofilms, combined with or without PA\(\beta\)N lysates, were detected by agar well diffusion method. The 1-day biofilms of *B. pseudomallei* K96243 were treated with CTZ, DOX and IMN with 0, 50 and 100 µg ml\(^{-1}\) PA\(\beta\)N, and the results are shown in Fig. 6. The inhibitory effect of each antibiotic at the MIC cut-off concentrations 32 µg ml\(^{-1}\) CTZ, 16 µg ml\(^{-1}\) DOX and 16 µg ml\(^{-1}\) IMN is shown in Fig. 6(a–c) and compared with 0, 50 and 100 µg ml\(^{-1}\) PA\(\beta\)N that was directly tested with *E. coli* ATCC 25922. The accumulation was not found in low-dose CTZ (32 µg ml\(^{-1}\), Fig. 6a upper right panel) but significantly found in 0.5 MBEC and MBEC. Inhibition of efflux transporter function by PA\(\beta\)N slightly increased the diameter of the inhibition, zone which represented the concentration of intracellular antibiotic in the treated biofilms. This evidence revealed that accumulated CTZ had been affected by efflux that blocked activity of PA\(\beta\)N (Fig. 6a, lower left and right panels). In the case of DOX-treated biofilm, it was found that the MIC cut-off concentration with or without PA\(\beta\)N did not give evidence of accumulation. Although 0.5 MBEC and MBEC of DOX were found to be accumulated, 50 and 100 µg ml\(^{-1}\) PA\(\beta\)N were found to have increased the inhibitory zone of the *E. coli* lawn when compared to those without PA\(\beta\)N (Fig. 6b, lower left and right panels). On the other hand, there was no evidence of intracellular IMN from biofilm lysate for all concentrations of treatment with or without PA\(\beta\)N. This is not surprising because previous MBEC results have shown that *B. pseudomallei* K96243 is highly resistant to IMN both with and without PA\(\beta\)N combined (Fig. 6c).

**Inhibition of efflux transporters leads to accumulation of intracellular CTZ and DOX**

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*B. pseudomallei* has a high potential for resistance to several kinds of antibiotics when it grows in the biofilm stage. Not only high biofilm production strains but also medium, low and biofilm-deficient mutant strains were able to develop resistance to several antibiotics when they were growing in biofilm-inducing conditions (Sawasdidoln et al., 2010). Those results indicated that the resistance may not be due to the biofilm itself but there might be other factors that are stimulated in the biofilm-growing conditions. Moreover, a previous report revealed that a high biofilm production strain gave lower diffusion of CTZ and trimethoprim/sulfamethoxazole than the biofilm-deficient strain (Pibalpakdee et al., 2012). Not only environmental survival factors but also the critical role of biofilm production in relapse of melioidosis have been reported (Limmathurosakul et al., 2014). In the case of *P. aeruginosa* PA01, the researchers found that the PA1874–PA1877-specific efflux pump encoding genes were expressed only in the biofilm stimulation condition, and these genes played a critical role in antibiotic resistance when *P. aeruginosa* was grown in the biofilm stage (Zhang & Mah, 2008). In this study, it was demonstrated that *P. aeruginosa* orthologous genes of biofilm-specific efflux pumps could also be found in *B. pseudomallei*. **Fig. 4. Susceptibility of *B. pseudomallei* strain K96243 to CTZ, DOX, IMN and PA\(\beta\)N. Susceptibility assays were performed in planktonic cells by the broth microdilution method; the shedding planktonic and biofilm cells were assayed by using CBD. The data are shown as MIC (white bars), MBIC (grey bars) and MBEC (black bars) for planktonic, shedding planktonic and biofilm cells. The dashed lines indicate the MIC cut-off point of each drug.**
The translated amino acid sequence of these candidate orthologues has been compared and predicted for subcellular localization (Moreno-Hagelsieb & Latimer, 2008; Yu et al., 2010). The results showed that BPSL1661, BPSL1664 and BPSL1665 are orthologues of PA1874, PA1876 and PA1877. The BPSL1661 was characterized as an extracellular protein. The translated products from BPSL1664 have been found to contain five ABC-transporter-related protein domains. In the case of BPSL1665, a biotin-requiring protein enzyme domain has been found to have HlyD family secretion protein and secretion signature protein domains. Therefore, the BPSL1664 and BPSL1665 might be the ABC transporter and RND transporter encoding genes. Other genes in B. pseudomallei, the RND efflux pumps, encoding genes located on chromosome 1, amrB, bpeA, bpeB and bpeR, were also selected, and it was found that amrB, the encoding gene in the parts of the AmrAB-OprA efflux system, was more highly expressed in B. pseudomallei 2-day biofilms. It was therefore considered that this efflux system might play a role in the adaptation of B. pseudomallei in mature biofilms when encountering the stressful conditions leading to antibiotic resistance.

According to a previous study, the amrAB-oprA system played an important role in aminoglycoside and macrolide resistance in planktonic B. pseudomallei (Moore et al., 1999). In the case of the BpeAB-OprB efflux system, this pump has been reported to play a role in spermidine transportation that is related to biofilm production and virulence of B. pseudomallei (Chan & Chua, 2010). In addition, another study of transposon insertion mutagenesis of bpeAB-oprB revealed that mutant strains of these genes showed hyper-susceptibility to macrodilide, clindamycin, fluoroquinolone, tetracycline, chloramphenicol and acriflavine but were not deficient in spermidine transportation ability when compared to their wild-type strain (Mima & Schweizer, 2010). In this study, it was found that bpeR (repressor gene for bpeAB-oprB) was decreased in relative expression levels in biofilms. The bpeA and bpeB, however, were also found decreased in relative expression levels. These results are related to a previous study that showed that the BpeAB-OprB efflux system did not play a role in biofilm formation but did play a role in resistance to a broad range of antibiotics (Mima & Schweizer, 2010). It might be possible that the conditions that were selected in the present study to grow biofilms did not induce the expression of these genes.

Recently, the work of Chin et al. (2015) compared global transcriptional levels of B. pseudomallei high and low biofilm-producing strains, U6 and U1, when grown in the biofilm stage. They found that 9.5% of genes involved in

Fig. 5. Effect of inhibition of efflux transporters on antibiotic resistance when growing B. pseudomallei in biofilm-inducing conditions. Susceptibility of the B. pseudomallei strain K96243 was determined in the shedding planktonic cells and biofilm and expressed as MBIC and MBEC values for CTZ (a), DOX (b) and IMN (c) combined with 0 μg ml⁻¹ PAβN (white bars), 50 μg ml⁻¹ PAβN (grey bars) and 100 μg ml⁻¹ PAβN (black bars) by the modified broth microdilution assay in the CBD. The dashed lines indicate the MIC cut-off point of each drug.
surface modification, surface component synthesis and cell wall biosynthesis were differentially expressed when compared between these two strains. From this phenomenon, they suggested that these genes might play important roles in the initial attachment of the biofilm formation process and be differentially expressed so that they might affect differences in the biofilm formation and virulence of each bacterial strain. The efflux transporter system is regarded as a fundamental system for bacterial strain. The efflux transporter system is regarded as a fundamental system for bacterial strain. The efflux transporter, was significantly increased in relative expression levels in biofilms.

In this current study, PAβN was used to inhibit efflux pump function, and susceptibility of B. pseudomallei biofilm cells was monitored. It was found that B. pseudomallei, in both planktonic and biofilm cells, is tolerant to PAβN itself as very high MIC, MBEC and MBEC levels were found at 512, 512 and >1024 µg ml⁻¹ PAβN, respectively. To study the effects of efflux pump inhibition and antibiotic resistance of B. pseudomallei biofilms while avoiding the direct effects of a high dose of PAβN, low concentrations of 50 and 100 µg ml⁻¹ PAβN were used. Interestingly, the presence of PAβN led to a two to four times decrease in MBIC. Moreover, in the case of MBEC, decreases of 16 times for CTZ and DOX, 200 times for CTZ and 32 times for IMN. The culture medium was removed and the biofilm was washed with sterile water. Lysis buffer was added to allow cell lysis. Cell lysates of each treatment were collected, and antibiotic concentrations were detected by the agar well diffusion method against sensitive E. coli ATCC 25922. The diameter of the inhibition zone corresponded to the amount of accumulated antibiotic inside the bacterial cells. The accumulation of antibiotic in B. pseudomallei biofilm lysate (with the resistance cut-off concentration, 0.5 MBEC and MBEC of each antibiotic) compared to their positive controls (antibiotics at resistance cut-off concentrations combined with PAβN were directly dropped into wells, upper left) is shown in each figure. Intracellular accumulation of antibiotics was found for CTZ and DOX but not found in IMN.

**Fig. 6.** Inhibition of efflux transporters led to accumulation of intracellular antibiotics. Antibiotic accumulation assays of CTZ (a), DOX (b) and IMN (c) with 0, 50 and 100 µg ml⁻¹ PAβN were performed. The 1-day biofilm of B. pseudomallei K96243 in MVBM was challenged for 24 h at 37 °C with MH broth containing antibiotics at resistance cut-off concentrations (32 µg ml⁻¹ CTZ, 16 µg ml⁻¹ DOX and 16 µg ml⁻¹ IMN), biofilm sub-eradication concentrations (0.5 MBEC: 512 µg ml⁻¹ CTZ, 64 µg ml⁻¹ DOX and 128 µg ml⁻¹ IMN) and biofilm eradication concentrations (MBEC: 1024 µg ml⁻¹ CTZ, 128 µg ml⁻¹ DOX and 256 µg ml⁻¹ IMN). The culture medium was removed and the biofilm was washed with sterile water. Lysis buffer was added to allow cell lysis. Cell lysates of each treatment were collected, and antibiotic concentrations were detected by the agar well diffusion method against sensitive E. coli ATCC 25922. The diameter of the inhibition zone corresponded to the amount of accumulated antibiotic inside the bacterial cells. The accumulation of antibiotic in B. pseudomallei biofilm lysate (with the resistance cut-off concentration, 0.5 MBEC and MBEC of each antibiotic) compared to their positive controls (antibiotics at resistance cut-off concentrations combined with PAβN were directly dropped into wells, upper left) is shown in each figure. Intracellular accumulation of antibiotics was found for CTZ and DOX but not found in IMN.
50 and 100 µg ml\(^{-1}\) PAβN, which led to a two-fold increased MBIC value. This antagonistic evidence of PAβN and IMN themselves might lead to different results in the susceptibility to these antibiotics in biofilms when efflux pumps were inhibited by PAβN. Moreover, there might be other different unknown mechanisms unrelated to efflux pumps during growth in biofilm conditions. When the CTZ and DOX concentrations in the treated cells were determined, however, an increase of intracellular antibiotics in biofilm cells treated with CTZ and DOX combined with PAβN was found. As \(B.\) pseudomallei was found to tolerate PAβN, the growth inhibition that was found in these experiments did not directly inhibit growth of \(B.\) pseudomallei cells with PAβN. The increase in susceptibility might be due to a malfunction of biofilm-related, antibiotic-resistant efflux transporters. This phenomenon could give indirect evidence about the role of efflux pumps in resistance to CTZ and DOX in \(B.\) pseudomallei biofilms. There might be other pumps, however, working to compensate or other unknown mechanisms which remain to be investigated. This might be an answer as to why \(B.\) pseudomallei was still resistant to IMN while efflux pumps were blocked in the current study. Recently, Lamers et al. (2013) revealed that PAβN can lead to an increase of outer membrane permeability in Gram-negative bacteria in a dose-dependent manner. An increased outer membrane permeability can lead to a loss of proton motive force which is a necessary driving force for RND efflux function. This evidence together with the nature of CTZ which inhibits cell wall synthesis by binding to the penicillin-binding protein 3, located on the bacterial cytoplasmic membrane (Schweizer, 2012), may explain why the study herein found an effect of PAβN on the resistance with low accumulation of intracellular CTZ. Because of the lower molar mass of IMN (299.347 g mol\(^{-1}\)) compared to CTZ (546.58 g mol\(^{-1}\)), the increase of the membrane permeability effect by PAβN might be a drawback to a decrease of intracellular IMN but not of CTZ. On the other hand, DOX is a member of the aminoglycoside class antibiotics that target protein synthesis by binding to small subunits of rRNA; thus, the loss of RND and other efflux functions might lead to accumulation of intracellular DOX.

Although expression levels of \(bpeA\) and \(bpeB\) were decreased in biofilm formation, relative expression levels of \(amrB\) were slightly increased. Nevertheless, the inhibitory effect of efflux pump inhibitors led to a more susceptible \(B.\) pseudomallei biofilm to CTZ and DOX and the accumulation of intracellular biofilm DOX. These results might lead to the conclusion that BPSL1665 or other effluxes could work to compensate for AmrAB-OprA and BpeAB-OprB and play a role in biofilm-related antibiotic resistance of \(B.\) pseudomallei. From this evidence, it is indicated that the efflux transport system may have played a role in some antibiotic resistance of \(B.\) pseudomallei biofilms. The resistance in biofilms is multifactorial, including the diffusion barrier and efflux transporter and the metabolic-related genes such as those for ethanol oxidation in \(P.\) aeruginosa (Beaudoin et al., 2012).

In conclusion, these current results showed that PA1874–PA1877 had orthologous genes on \(B.\) pseudomallei chromosome 1 which included BPSL1661, BPSL1664 and BPSL1665. The BPSL1665 gene was found more highly expressed during biofilm induction growth conditions and might be related to antibiotic resistance in biofilms. PAβN, the universal efflux pump inhibitor, led to a decrease of CTZ and DOX resistance in biofilms and led to an intracellular accumulation of CTZ and DOX in biofilm cells. This evidence supports the resistance of \(B.\) pseudomallei to drugs of choice for melioidosis treatments when growing in biofilm induction conditions is related to efflux pumps.

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