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BPSL1665 does not play a role in ceftazidime resistance during biofilm growth of *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis, a serious infectious disease affecting both human and animals. Ceftazidime (CAZ) is a drug of choice. However, the bacterium in biofilm form becomes resistant to CAZ with mechanisms remain unclear. *B. pseudomallei* BPSL1665 is orthologue with PA1877, an efflux pump gene in *Pseudomonas aeruginosa* that participated in biofilm-related antibiotics resistance. BPSL1665 expressed higher in biofilm form, and a universal efflux pump inhibitor, phenylalanine arginine β-napthylamide (PAβN), could increase the CAZ sensitivity of the bacterium. *B. pseudomallei* BPSL1665 mutant therefore was constructed and tested for CAZ and Ciprofloxacin (CIP) susceptibility. The minimal inhibitory concentration (MIC) of wild type and the mutant of planktonic against CAZ and CIP were 4 and 2 μg/mL. The Minimum Biofilm Inhibitory Concentration (MBIC) and the Minimum Biofilm Eradication Concentration (MBEC) against CAZ of wild type and the mutant were 32 and >1024 μg/mL. The MBIC and MBEC against CIP of the wild type and the mutant were 16 and 512, and 8 and 512 μg/mL. When 50 μg/mL of PAβN was combined with each antibiotic, the titer of MBIC was reduced to 2 μg/mL, however, the MBEC against CAZ did not change. For CIP, the MBIC and the MBEC were not changed. Nevertheless, the BPSL1665 mutant showed all responses similar to its wild type. Recently, BPSL1665 has been annotated as type I secretion membrane fusion, HlyD family protein. PAβN may influence other efflux pumps that further study may help improve the *B. pseudomallei* biofilm treatment.

Keywords: Antibiotic resistance, Biofilm, Efflux pump, Melioidosis, Mutant

1. Introduction

Burkholderia pseudomallei is the etiologic agent of melioidosis that was first discovered in 1911 [1]. The bacterium is a Gram-negative bacillus reported to be endemic in Southeast Asia and northern Australia with sporadic cases in tropical and sub-tropical countries [2]. *B. pseudomallei* infection was found to be a major cause of bacterial pneumonia and acute bacterial sepsis [3]. Long-term treatments are required for intravenous and oral phases. Accurate diagnostic tests with effective therapeutic lead to improve outcomes and lower the mortality rate [4]. The failure to eradicate the infection causes relapse melioidosis. Biofilm formation of *B. pseudomallei* is related to relapse of melioidosis and has been shown highly resistance to a wide range of ceftazidime (CAZ), the drug of choice, than their planktonic forms [5]. Biofilms can be simply and broadly defined as communities of microorganisms that are attached to a surface [6]. They were also reported as a barrier that causes antibiotic resistance, thus making the therapeutic drugs insufficient in the treatment of chronic infections [7]. Antibiotic resistance mechanisms in *B. pseudomallei* include cell exclusion, Beta-lactamase deactivation [8], target site loss, and the function of efflux pumps [9]. Efflux pumps in Gram-negative bacteria are a subset in the resistance-nodulation-cell-division (RND) superfamily, their function can cause multiple drug resistance phenotypes in several pathogens [9].

The analysis of *B. pseudomallei* K96243 genome sequence indicated the presence of at least 10 operons encoded for the RND efflux pump component on both chromosome 1 and chromosome 2. There are 3 RND efflux pumps involved in multidrug resistance in *B. pseudomallei* that have been characterized [10]. The AmrAB-OprA and BpeAB-OprB pumps similarly excrete both aminoglycoside and macrolide. The third one, BpeEF-OprC, was demonstrated to efflux trimethoprim and chloramphenicol. These complexes were reported to be responsible for the exporting of these drugs out of the bacterium. A novel efflux pump in *Pseudomonas aeruginosa* PA14 was reported to overexpress only during the biofilm formation process [11]. It is a group of RND efflux pump composts of genes that function together, and the complete deletion of PA1874-PA1877 genes encoded in the efflux pump results in an increase in sensitivity to tobramycin, gentamicin, and ciprofloxacin, specifically when this mutant strain is growing in biofilm condition [11]. *B. pseudomallei* and *P. aeruginosa* are closely related [12], and the orthologues genes of PA1874-1877 efflux pump were BPSL1661-BPSL1665 genes in *B. pseudomallei* chromosome 1. Only BPSL1665 expressed higher in the biofilm-induced condition, of which when it became resistant to ceftazidime [13]. Moreover, a universal efflux pump inhibitor (phenylalanine arginine β-napthylamide, (PaβN)) could increase sensitivity to CAZ in this condition.

Even though the results were convincing that the BPSL1665 may involve in the drug resistance during biofilm stage, the broad inhibition activity of PAβN might lead to some argument. Therefore, *B. pseudomallei* BPSL1665 knock out was constructed by allele replacement method and evaluated the CAZ, the drug of choice and ciprofloxacin (CIP), a fluroquinolone antibiotic, for their resistance in the biofilm-induced condition.

2. Materials and methods

2.1 Bacterial strains

B. pseudomallei strain K96243 is the first sequenced strain isolated from a patient admitted at Khon Kaen Hospital, Khon Kaen province, Thailand [14]. This strain is sensitive to CAZ, which is the therapeutic drug of melioidosis. The culture media for *B. pseudomallei* were Ashdown's selective agar (ASH), Luria-Bertani medium (LB), Muller-Hinton broth (MHB) and Modified Vogel and Bonner's medium (MVBM). Media for *E. coli* DH5α harboring plasmid DNA, pGemT® easy vector, Promega, USA, and pEXkm5, kindly provided by Professor Herbert P Schweizer, Colorado State University, USA, were augmented with 35 µg/mL kanamycin (KM). *E. coli* S17-1λpir was grown in LB medium containing 50 µg/mL trimethoprim (TMP). For merodiploid selection of chromosomally integrated pEXKm5DΔBPSL1665 recombinant plasmid in *B. pseudomallei* K96243, cells were plated on LB agar supplemented with 500 µg/mL KM and 50 µg/mL of X-Gal (Thermo Fisher Scientific, USA) [15]. All bacterial strains were grown at 37°C.

2.2 Construction of BPSL1665 deletion mutant (BPSL1665 mutant) by allelic replacement method

The upstream region of PBSL1665 gene was amplified from *B. pseudomallei* K96243 genomic DNA using primers: P1 (5'-TTACTCTCGAGGGTGGCGAACGTGAATCTGA) and P2 (5'-AGTGCTGCCGAAA-TCACCCTGACATCCCCTTTGCGCCGG) to obtain the upstream fragment (703 bp) and P3 (5'-CCGGCGCAAAGGGGATGTCAGGGTGATTTCGGCAGCACT) and P4 (5'-TATCAGAATTCAGC-ACGAGTTCAATCGCACA) to obtain the downstream fragment (694 bp) of the gene. The 703 and 694 bp amplicons were ligated by using overlap extension polymerase chain reaction (PCR) with designed primers to obtain 1,397 bp with restriction enzyme EcoRI on one end and XhoI on another end [16]. The PCR product (ΔBPSL1665) was purified from agarose gel (QIAquick gel extraction kit (QIAGEN, Germany), ligated to TA cloning vector (pGemT® easy vector system, Promega, USA) using 1:3 molar ratio of PCR product (to the vectors and transformed into *E. coli* DH5α by 1:10 molar ratio of plasmid DNA (pGemT-ΔBPSL1665) to competent cells using the electroporator (Bio-Rad, USA). Transformants were identified on selective Luria-Bertani (LB) agar plates containing X-gal, IPTG, and 35 µg/mL KM (Sigma-Aldrich, USA).

The pGemT-ΔBPSL1665 was extracted, digested with EcoRI and XhoI, and ligated into pEXKm5 vector [15]. The *E. coli* DH5α harboring recombinant plasmids (pEXKm5ΔBPSL1665) were selected by blue-white selection supplemented with 35 µg/mL KM and further transformed into *E. coli* S17-1λpir (kindly provided by Prof Ben Adler, Monash University, Australia). Bi-parental conjugation was used to deliver recombinant plasmid from *E. coli* S17-1λpir (*E. coli* S17-1λpir/pEXKm5ΔBPSL1665) as a donor contained the pir gene (λpir) with chromosomally integrated conjugal transfer functions to the recipient, *B. pseudomallei* K96243.

For bi-parental conjugation, the donor in LB supplemented with 35 µg/mL KM and the recipient in LB broth were added into a microcentrifuge tube containing 600 µL of 10 mM $MgSO_4$ and pelleted the cells by centrifugation at 12,000 rpm for 5 min. The conjugation mixture of cells was washed by PBS, pH 7.4, and re-suspended in 1.5 mL of 10 mM $MgSO_4$ before being applied onto a cellulose acetate membrane that was placed on LB agar plate containing 4% glycerol and 10 mM $MgSO_4$ and incubated at 37°C for 18 h. Bacterial cells were then transferred to a new centrifuge tube with LB, centrifuged to obtain cell pellet, re-suspended in 500 µL sterile

phosphate-buffered saline (PBS), and spread on LB plates containing 4% glycerol, 5 μg/mL GM, 1 mg/mL KM and 50 μg/mL X-gal and incubated at 37°C for 24 h. The *B. pseudomallei* BPSL1665 mutant appeared as white colonies on the X-gal plate was confirmed by colony PCR for the products of 1,397 bp and DNA sequencing using P1 and P4 primers designed to amplify gDNA flanking the BPSL1665 region of *B. pseudomallei* K96243 (Pacific Science, USA).

2.3 Bacterial colony morphology and growth curve analysis of B. pseudomallei BPSL1665 mutant

Colony morphology of *B. pseudomallei* strain K96243 wild type and BPSL1665 mutant were observed on Ashdown's agar. Their growth curves were observed by growing each one in LB broth at 37°C and measuring the optical density (OD) at 600 nm every 2 h.

2.4 Antibiotic susceptibility testing

The minimal inhibitory concentration (MIC) for planktonic form was determined by standard broth microdilution method [17]. A fifty-microliters of two-fold serial dilution of antibiotics in Mueller-Hinton broth; for a final concentration of 1-1024 μg/mL of CAZ and 0.5-256 μg/mL of CIP were prepared in 96-well microtiter plate and mixed with 50 μL of *B. pseudomallei* K96243 wild-type or BPSL1665 mutant overnight culture that were adjusted to a final concentration of 1×10^5 CFU/mL in Müller Hinton broth (MHB) and incubated at 37°C for an overnight. The lowest concentration of antibiotics that inhibited bacterial growth as observed by the OD 600 < 0.1 was considered as the MIC.

The antibiotic resistance in biofilm form of *B. pseudomallei* K96243 wild-type and BPSL1665 mutant strain in the presence or absence of 50 μg/mL efflux pump inhibitors PaβN was done by growing the bacterium in MVBM broth for 18 h followed by adjusted bacterial density to 1×10^7 CFU/mL before inoculated into fresh MVBM to form 2-day biofilm in the Calgary Biofilm Device (CBD) as previously described [18]. The biofilms on the pegs were transferred to the 96-well microtiter plate containing specified antibiotics; 1-1024 μg/mL of CAZ, 1-1024 μg/mL of CIP and/or PAβN in MHB broth and incubated at 37°C for 24 h. The Minimum Biofilm Inhibitory Concentration (MBIC), defined by the minimum concentration of antibiotic that inhibits the growth of the bacterium in the biofilm forms, and the Minimum Biofilm Eradication Concentration (MBEC) of antibiotic that eradicates the bacterium in the biofilm was determined in recovery media [18]. The OD 600 < 0.1 measured by a 96-well microtiter plate reader was considered as the antibiotic inhibition dilution. All the experiments were done in 3 biological replicates.

2.5 Statistical analysis

The significant differences of bacterial growth between *B. pseudomallei* K96243 and BPSL1665 mutant were analyzed by the Mann-Whitney U test using the SPSS software, version 28.0 (Chicago, IL); $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

3. Results

3.1 Construction of B. pseudomallei BPSL1665 mutant

The PCR products from the upstream (703 bp) and downstream (694 bp) of the BPSL1665 were successfully amplified and ligated to obtain the 1,397 bp fragment (Figure 1A). This fragment was then subcloned into pEXKm5 vector and transformed into *E. coli* S17-1λpir (donor strain) as confirmed by PCR using primers that flanked the gene (Figure 1B). The allelic replacement was successed as confirmed by PCR. Amplification using a pair of primers that flanking BPSL1665 against *B. pseudomallei* K96243 wild type provided a 2,753 bp PCR product (Figure 1C) while that of a white colony on the selective medium of *B. pseudomallei* BPSL1665 mutant gave a 1,397 bp PCR product (Figure 1D). The mutant colony was further confirmed by PCR and the gene region was also sequenced. From the nucleotide sequences comparison between *B. pseudomallei* K96243 in the NCBI database and the sequences flanking the BPSL1665 gene in the mutant, it indicated that the whole BPSL1665 gene sequence was deleted that confirming the success of *B. pseudomallei* BPSL1665 mutant construction (Figure 2).

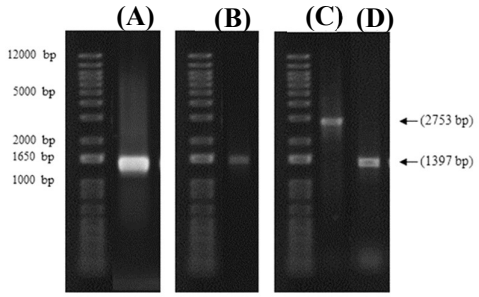

Figure 1 Agarose gel electrophoresis showed the PCR products from each step in the *B. pseudomallei* BPSL1665 mutant construction using an allelic replacement system. The upstream and downstream flanking regions of BPSL1665 gene were amplified and assembled to obtain 1,397 bp fragments (A), a PCR product from amplification of the *E. coli* S17-1λpir harboring recombinant plasmid with the 1,397 bp fragment (B), PCR products of 2,753 bp obtained from the amplification of the BPSL1665 gene in *B. pseudomallei* K96243 wild-type (C) and PCR product of 1,397 bp from amplification of truncated BPSL1665 in *B. pseudomallei* BPSL1665 mutant (D): Lane M, 1 KB plus DNA ladder.

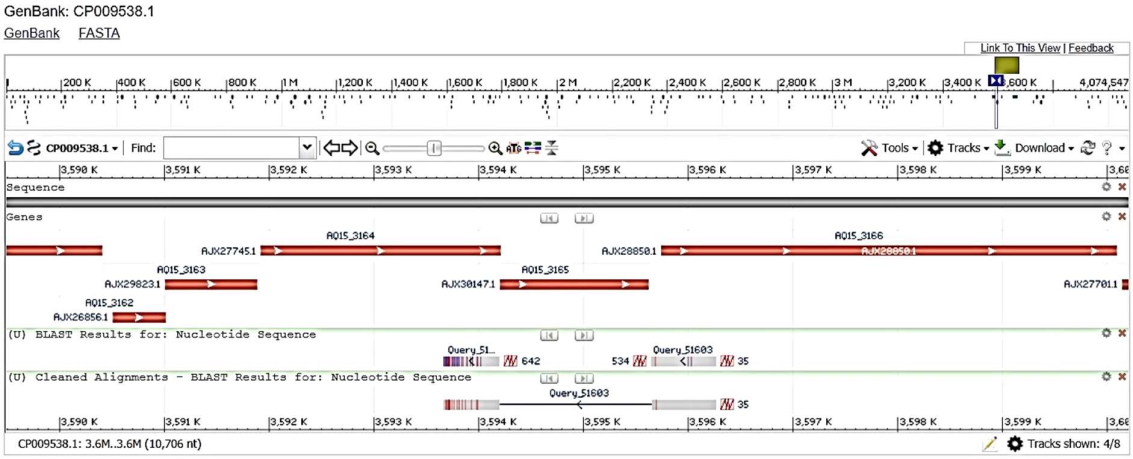

Figure 2 The comparison of sequence from PCR amplification of BPSL1665 gene sequence in *B. pseudomallei* BPSL1665 mutant with the chromosome 1 of *B. pseudomallei* K96243 wild type on the National Center for Biotechnology Information (NCBI) database. The nucleotide sequence from the PCR product (query_51603) in the red box and the lower picture indicated the deletion of BPSL1665 sequence.

3.2 Some phenotypes of B. pseudomallei BPSL1665 mutant

3.2.1 Colony morphology, growth, and biofilm formation

Colonies of the BPSL1665 mutant appeared as wrinkled purple colonies with irregular edges like *B. pseudomallei* morphotype I [18] and the same as its wild type (Figure 3A). The growth curves of *B. pseudomallei* K96243 and the mutant were also similar (Figure 3B). The amount of 2-day biofilm formation of the mutant was also similar to its wild type (data not shown).

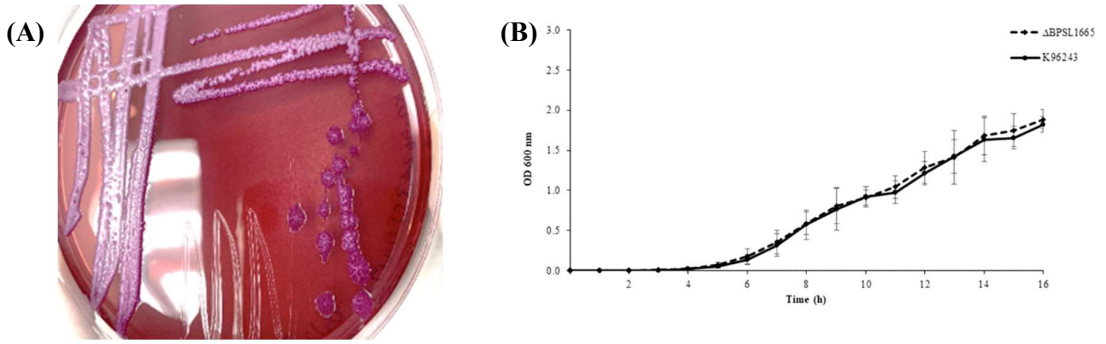

Figure 3 Colony morphology and growth curve of *B. pseudomallei* K96243. The colony morphology of *B. pseudomallei* BPSL1665 mutant appeared as wrinkled purple on Ashdown's agar, the same as its wild type (A). The growth curve of *B. pseudomallei* K96243 wild-type (solid line) and BPSL1665 mutant (dash line) grew in LB broth (B). The X axis is time in hour, and the Y axis is the OD at 600 nm. The error bars represent an average of 3 biological replicates.

3.3 The antibiotic susceptibility and the effect of efflux pump inhibitor

The antibiotic susceptibility of *B. pseudomallei* wild type and BPSL1665 mutant against CAZ, CIP, or combination with 50 µg/mL PAβN were evaluated in both planktonic and biofilm forms. The planktonic form of the wild-type and mutant strains showed MIC of CAZ as 4 µg/mL and CIP at 2 µg/mL, while the MBC of CAZ and CIP was 16 µg/mL in both wild type and the mutant (Table 1).

In the biofilm form, the wild-type and mutant strains showed MBIC of CAZ to be 32 µg/mL and CIP as 16 µg/mL in wild type and 8 µg/mL in the mutant. For the MBEC of CAZ, both wild type and the mutant showed at least 1024 µg/mL as the turbidity of the bacterial culture was observed until the last dilution concentration of 1024 µg/mL. The MBEC of CIP was 512 µg/mL for both strains (Table 2).

When 50 µg/mL of PAβN efflux pump inhibitor was combined with CAZ, the MBIC of both wild type and the mutant was decreased to 2 µg/mL, however, the MBEC was still at least 1024 µg/mL (Table 2). When PAβN was combined with CIP, the MBIC of both strains remained unchanged or just 1 dilution less and MBEC values were all unchanged (Table 2).

Table 1 The antibiotic susceptibility of *B. pseudomallei* K96243 wild type and the BPSL1665 mutant in the planktonic form.

Strain	CAZ		CIP	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
K96243	4	16	2	16
BPSL1665 mutant	4	16	2	16

Table 2 The effect of PAβN on antibiotic susceptibility of *B. pseudomallei* K96243 wild-type and BPSL1665 mutant in the biofilm form.

Strain	CAZ		CAZ+PaβN		CIP		CIP+PaβN	
	MBIC (µg/mL)	MBEC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)
K96243	32	>1024	2	>1024	16	512	8	512
BPSL1665 mutant	32	>1024	2	>1024	8	512	8	512

4. Discussion

Several antibiotics resistance in *B. pseudomallei*, including β-lactams, aminoglycosides, macrolides, and polymyxins are encoded in its genome. It is generally susceptible to CAZ, tetracycline, and doxycycline, thus limiting the choice of antimicrobials for the effective treatment of melioidosis [5]. The efflux pump-mediated drug resistance is one of the important mechanisms reported in *Burkholderia* spp. [10]. Two RND pumps in *B. pseudomallei* were responsible for either intrinsic or acquired resistance to aminoglycosides, macrolides,

chloramphenicol, fluoroquinolones, tetracyclines, trimethoprim, and trimethoprim plus sulfamethoxazole [10]. However, none of them were characterized in the biofilm-induced condition as reported in *P. aeruginosa*.

The BPSL1665 that ortholog with PA1877 (HlyD) in *P. aeruginosa* efflux pump [13] (34.24% protein sequence identity with significant E-value = 1×10^{-62} by Blastp) and significantly expressed higher in the biofilms condition was successfully clean deleted to obtain *B. pseudomallei* K96243 BPSL1665 mutant. The deletion of BPSL1665 showed no effect on its growth, colony morphology on Ashdown's selective medium, and biofilm formation when compared to its wild type. The MBIC of CAZ in biofilm form for both *B. pseudomallei* K96243 and its BPSL1665 mutant was 8-fold higher than the MIC of planktonic form as previously reported [13]. However, the combination of CAZ and 50 μg/mL PAβN to treat *B. pseudomallei* K96243 wild-type and BPSL1665 mutant strains under biofilm-induced conditions reduced only MBIC from 32 μg/mL to 2 μg/mL but did not change their MBEC. The PaβN is a short synthetic peptide that acts as a broad-spectrum competitive-type efflux pump inhibitor [19]. The concentration of antibiotic is retained inside the bacterial cell due to the cause of the efflux pump inhibitor may cause only a static effect to the bacterium, as observed by MBIC, but not a bactericidal effect, as observed by MBEC. As wild type and the BPSL1665 mutant showed all CAZ susceptibility in the same way, the BPSL1665 gene should not be responsible for the CAZ resistance in the biofilm-induced condition in *B. pseudomallei* as previously suspected. *B. pseudomallei* K96243 genome analysis [20] indicated at least 10 operons encoded for RND efflux pumps. The reduction of CAZ susceptibility observed previously and, in this study, may be contributed to the inhibition action against some efflux pumps in *B. pseudomallei*. One more point to be mentioned here is that the reduction of MBEC of CAZ that was not observed in this study may be due to the diversity of the growth phase and the complexity of components in the biofilm form that interfered with the random effect of the inhibitor to the outcome of MBEC.

Zhang et al. [12] reported the PA1874-1877 efflux pump operon deletion in *P. aeruginosa* could increase the susceptibility to tobramycin, gentamycin, and ciprofloxacin. We therefore further investigated the ciprofloxacin susceptibility of *B. pseudomallei* K96243 wild-type and BPSL1665 mutant. The planktonic form of *B. pseudomallei* K96243 BPSL1665 mutant showed the same susceptibility to CIP at low concentrations (2 μg/mL) as previously reported [21]. In addition, the MBIC and MBEC of CIP did not change when PaβN was combined in both *B. pseudomallei* K96243 wild-type and the mutant. PaβN was reported to block the excretion of several aminoglycosides and β-lactam type antibiotics in doxycycline (DOX)-selected strains of *B. thailandensis* [22]. CIP is a fluoroquinolone antibiotic whose mechanisms of resistance reported in *B. pseudomallei* are through efflux activity and target mutation [23]. The absence of BPSL1665 did not change the susceptibility on CIP in the presence of PAβN in the biofilm form. Nevertheless, we can only conclude here that the PaβN showed no effect to the CIP susceptibility in the biofilm form of both wild-type and BPSL1665 mutant.

The sequences of BPSL1665 gene (BPS_RS08815) (1,416 bases) from NCBI database (https://www.ncbi.nlm.nih.gov/) recently was predicted to be the toxin-related protein and has been characterized as HlyD, a hemolysin D family type I secretion periplasmic adaptor unit (NC_006350.1) which is a member of Membrane fusion Protein (MFP) family [24]. This adaptor protein is localized in the inner membrane and functions together with BPSL1664, ATP-binding cassette domain-containing protein (ABC transporter) and BPSL1666 which is tetratricopeptide repeat region. From these data, the BPSL1665 function is not the same as its homolog gene in *P. aeruginosa* and may be an inner membrane protein in the periplasm of the T1SS. The study using the concept of orthologous gene from one bacterium to another here is another example that sequences derived from the same origin may not have the same function. On the other hand, *P. aeruginosa* MexAB-OprM that widely expressed and responsible for intrinsic resistance to numerous antibacterial compounds, including aminoglycosides and macrolides [10,25,26] are not homolog with *B. pseudomallei* BpeAB-OprB, of which is a multidrug efflux pump that related to the antimicrobial resistance to aminoglycosides and macrolides [27].

5. Conclusion

Even though the BPSL1665 gene in *B. pseudomallei* is an orthologue to PA1877 gene in *P. aeruginosa* strain PA01 of the efflux transporter encoding genes for biofilm-related antibiotic resistance, it was not responsible for the CAZ and CIP resistance in *B. pseudomallei* K96243 biofilm form. The PaβN, a competitive-type efflux pump inhibitor, could increase CAZ susceptibility as observed in MBIC but not the MBEC and showed no effect on the CIP susceptibility.

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