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Contribution of EmrAB efflux pumps to colistin resistance in *Acinetobacter baumannii*[§]

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Efflux pumps play an important role in antimicrobial resistance for *Acinetobacter baumannii*. However, the function of the Emr pump system and the relationship between Emr and drug resistance has not been characterized in *A. baumannii*. In this study, four possible groups of *emr*-like genes were found by searching a genome database. Among them, A1S_1772 (*emrB*) and A1S_1773 (*emrA*) were demonstrated to be co-transcribed as a single operon. Moreover, during osmotic stress, A1S_1772 showed the largest change in gene expression compared to the other *emrB*-like genes, and deletion of A1S_1772 (AB Δ*emrB*) significantly slowed cell growth in 20% sucrose. Using a phenotypic microarray analysis, the AB Δ*emrB* mutant was more susceptible to colistin and nafcillin, paromomycin, spiramycin, and D,L-serine hydroxmate than the wild type. The spot assay, time kill assay and minimal inhibition concentration determination also indicated that the wild type could tolerate colistin better than the AB Δ*emrB* mutant. Finally, the increased expression levels of all *emrB*-like genes, including A1S_0775, A1S_0909, A1S_1772, and A1S_1799, in colistin resistance-induced *A. baumannii* further supported the possible involvement of the *emrB* genes in *A. baumannii* colistin resistance. Together, the Emr pump systems in *A. baumannii* contribute to adaptation to osmotic stress and resistance to colistin.

Keywords: efflux pump, colistin, antimicrobial resistance, *Acinetobacter baumannii*

Introduction

Many reports have emphasized the role of efflux pumps in multidrug resistance in bacteria (Poole, 2002). Efflux pumps are required to work together with the outer membrane to allow antimicrobials to be expelled out of the cell through the low-permeability outer membrane and to re-enter the cell (Hooper, 2005). An increased efflux of antibiotics reduces drug accumulation within the bacterial cells and increases the minimal inhibitory concentration. In Gram-negative pathogens, efflux pumps that provide clinically significant levels of multidrug resistance are generally composed of three components: the inner membrane transporters, periplasmic proteins, and outer membrane channels. The transporters belong to one of the three protein superfamilies, including resistance-nodulation-division (RND), ATP-binding cassette (ABC), and major facilitator (MF), whereas the periplasmic proteins belong to the membrane fusion protein (MFP) family. The outer membrane channel is exemplified by the *Escherichia coli* TolC protein. The efflux pumps span the entire two-membrane envelope of Gram-negative bacteria and expel toxic molecules out of the cell. The architecture of efflux pumps is expected to vary significantly because of the structural diversity of the inner membrane transporters (Tikhonova *et al.*, 2009).

In *A. baumannii*, four categories of efflux pumps are related to antimicrobial resistance, including RND, MF, multidrug, and toxic compound extrusion (MATE) and small multidrug resistance (SMR) family of transporters (Lin and Lan, 2014). Using a comparative genomics approach, 46 open reading frames (ORFs) that are putatively associated with resistance to antimicrobials were identified in the *A. baumannii* AYE strain (Fournier *et al.*, 2006). Of these 46 ORFs, 32 ORFs were associated with the RND family, seven with the MF family, two with the MATE family and one with the SMR family. AdeABC is a member of the RND family of transporter and is the best characterized efflux pump in *A. baumannii* (Magnet *et al.*, 2001; Ruzin *et al.*, 2007). Inactivation of other RND type efflux pumps, including AdeDE (Chau *et al.*, 2004), AdeFGH (Coyne *et al.*, 2010), and AdeIJK (Damier-Piolle *et al.*, 2008), demonstrated their contribution to multidrug resistance in *A. baumannii*. Moreover, a number of MF efflux pumps were characterized in *A. baumannii* as conferring resistance to different types of antibiotics. For example, TetA has been found to be associated tetracycline resistance (Ribera *et al.*, 2003), MdfA with ciprofloxacin and chloramphenicol resistance (Vila *et al.*, 2007), CraA (Roca *et al.*, 2009), and CmlA (Coyne *et al.*, 2011) with chloramphenicol resistance, and AmvA with erythromycin resistance (Rajamohan *et al.*, 2010).

In contrast to the above-mentioned efflux pumps, the functions of other transporter proteins, such as EmrA and EmrB, are mostly unknown in *A. baumannii*. The *emrB* gene related to multidrug resistance was sequenced from *E. coli* and its gene product (EmrB) was homologous to members of the

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MF family (Lomovskaya and Lewis, 1992). In addition, EmrR was found to act as a negative regulator of EmrAB (Lomovskaya *et al.*, 1995). *E. coli* strains carrying a plasmid containing the *emrA* and *emrB* genes showed increased resistance to toxic compounds, including carbonylcyanide m-chlorophenylhydrazone (CCCP), 2-chlorophenylhydrazine hydrochloride (CHH), tetrachlorosalicylanflide (TSA), nalidixic acid, and phenylmercury acetate (PMA) (Lomovskaya and Lewis, 1992). Moreover, VceAB of *Vibrio cholera* shows a high degree of similarity with EmrAB in *E. coli* (Colmer *et al.*, 1998). A *vceB* mutant is more sensitive to CCCP, PMA, pentachlorophenol (PCP), nalidixic acid and deoxycholate than its parental strain. Therefore, bacterial efflux pumps not only export antibiotics but also other substances, such as dyes, detergents and host-derived antimicrobial agents. Besides, the accumulating evidence shows that multidrug-resistance efflux pumps have roles in bacterial pathogenicity and in allowing bacteria to survive in their ecological niche (Piddock, 2006). The aim of this study is to facilitate the functional analysis of EmrA and EmrB in *A. baumannii* to define a potential target to combat multidrug resistant *A. baumannii*.

Materials and Methods

Identification of *emrA*-like and *emrB*-like genes

By searching of the GenBank sequence database (http://www.ncbi.nlm.nih.gov/genbank), four pairs of *emrA*-like and *emrB*-like genes were identified from *A. baumannii* genome sequences. Using Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), EMBOSS (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), and Prosite (http://prosite.expasy.org/), the sequences of *emrA* and *emrB* genes from other bacterial species were aligned with the predicted *A. baumannii emrA*- and *emrB*-like genes, and the functional domains were determined.

Co-transcription of *emrA/emrB* by reverse transcription (RT)-PCR

Co-transcription of the *emrA* and *emrB* genes was demonstrated by RT-PCR. Briefly, the overnight culture of *A. baumannii* strain ATCC 17978 was sub-cultured in fresh LB medium to grow to mid-log phase. The cell pellets were harvested by centrifugation. RNA extraction and cDNA synthesis were performed as described in our previous study (Lin *et al.*, 2014). For PCR, the primer pair 1773-int_F and 1772-int_R were used (Table 1). As an endogenous control, a primer pair for 16S rRNA was also used.

Expression of *A. baumannii emrB*-like genes under osmotic stress

The expression levels of *emrB*-like genes were compared in cells grown with and without an osmotic stress condition

Table 1. Bacterial strains, plasmids and primers used in this study

Strain		Relevant feature(s)	Source or reference
A. baumannii strains	ATCC 17978	Wild-type strain	ATCC
	ΔemrB	Derived from ATCC 17978. A1S_1772 mutant	This study
	17978CR	Induced colistin resistant ATCC 17978	This study
E. coli strains	XL1 blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Stratagene
	S17-1 (ATCC 47055)	*thi pro hsdR hsdM recA*[*RP42-Tc::Mu-* Km::*Tn7* (Tp^{r}Sm^{r})Tra^{+}]	ATCC
	S17-1 A1S_1772	S17-1 carrying plasmid pEX18Tc-1772	This study
Plasmids		**Relevant feature(s)**	**Source or reference**
pEX18Tc-1772		pEX18Tc containing A1S_1772 upstream and downstream fragments	This study
Primers		**Sequence (5′ to 3′)**	**Source or reference**
1772-up-*Sal*I_F		TATAGTCGACCAAGCCGATGTCACTTG	This study
1772-up-*Bam*HI_R		TATCGGATCCCATGGCAGCAAAAATTGC	This study
1772-dw-*Kpn*I_F		ATAGGGTACCAGAAATGAATATGTAGCGAT	This study
1772-dw-*Sac*I_R		ATTAGAGCTCGAGAGTCAACCATTGATTAG	This study
1773-int_F		AAACCTGGAATGTCGGTGAG	This study
1772-int_R		ATAGCCAAATGCGAGACCAC	This study
emrB_F		CCAGAACCAAAACGCTCATT	This study
emrB_R		ATAGCCAAATGCGAGACCAC	This study
emrB_5_F		GTGTTTTTAGTGTCCTGCTG	This study
emrB_3_R		GGTCAGATGCATCACACC	This study
qA1S_0775_F		AGCGATGTACGGCCTTCAAA	This study
qA1S_0775_R		GGCCGCCGGCAAAG	This study
qA1S_0909_F		CTTATGCCACTCAGCCAAACC	This study
qA1S_0909_R		CCTGCGCATGCTTTTCTTG	This study
qA1S_1772_F		GCGGGATGATTCCGACTTC	This study
qA1S_1772_R		TGAGCGTTTTGGTTCTGGAAA	This study
qA1S_1799_F		GCAGATGGTACCGAACGTGTT	This study
qA1S_1799_R		TGTGCCTGAATCGCAGAATTT	This study
q16S rRNA_F		AGCATTTCGGATGGGAACTTTA	Lin *et al.* (2014)
q16S rRNA_R		GTCGTCCCCGCCTTCCT	

(20% sucrose) by qRT-PCR as previously described (Lin *et al.*, 2014). The primers were designed specifically for the individual *emrB*-like genes (Table 1). The 16S rRNA transcript was used as an endogenous control. StepOne Software v2.1 (Life Technologies) was used for the data analysis.

Construction of *emrB* gene deletion (AB Δ*emrB*) mutant

To assess gene function, a mutant with an *emrB* (A1S_1772) gene deletion was constructed from *A. baumannii* ATCC 17978 as previously described (Sugawara and Nikaido, 2012), and the procedure is outlined in Supplementary data Fig. S1.

Comparison of the osmotic stress response and cell growth between the wild type and AB Δ*emrB* mutant

An overnight culture of the wild type and the AB Δ*emrB* mutant was sub-cultured (1:100 dilution) in 25 ml of fresh LB medium and grown at 37°C with shaking (220 rpm). The cell growth was monitored hourly at an optical density of 600 nm (OD_{600}).

To determine the role of the *emrB* (A1S_1772) gene in response to osmotic stress, the cell growth of the wild type and AB Δ*emrB* mutant were compared in LB broth with or without 20% sucrose (at 37°C with 220 rpm shaking).

Phenotypic microarray assay

To examine the role of the EmrAB transporter in antimicrobial susceptibility in *A. baumannii*, the wild type and the AB Δ*emrB* mutant were assayed using the Biolog Phenotype MicroArray™ (PM) system (Biolog) as previously described (Lin *et al.*, 2015). The microplates PM11C and PM12B containing a total of 48 chemical compounds and antibiotics were used.

Minimal inhibitory concentration (MIC) determination, spot assay and time kill assay

To confirm the results of phenotype microarray, spot assays were performed. An overnight culture of *A. baumannii* ATCC 17978 and the AB Δ*emrB* mutant was sub-cultured in 3 ml LB broth (initial OD_{600} ~0.3) and grown to mid-log phase. The cell pellets were harvested by centrifugation and were re-suspended in 1 ml phosphate buffered saline (PBS). A ten-fold serial dilution of the bacterial suspension was then prepared using PBS. The bacteria were then spotted (10 µl per spot) onto the LB agar plates containing different concentrations of antimicrobial agents and were incubated at 37°C for 24 h. To determine the MIC of antimicrobials, a broth microdilution method according to the 2014 CLSI guidelines was used (CLSI, 2014).

To further compare the effect of EmrB on colistin susceptibility, a time-kill assay was performed using *A. baumannii* ATCC 17978 and the AB Δ*emrB* mutant as previously described (Lin *et al.*, 2014).

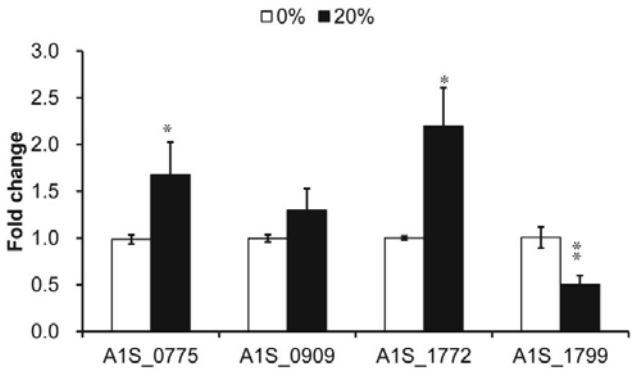

Fig. 1. Relative transcription level of the four *emrB*-like genes in *A. baumannii* by qRT-PCR with or without 20% sucrose (37°C, 220 rpm). The cells were grown in LB agar with or without 20% sucrose. The relative transcription levels were determined by comparison with an internal 16S rRNA control. The results were from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

Results

Identification of *emrA*-like and *emrB*-like genes in *A. baumannii*

In a search of the GenBank database, four pairs of genes currently annotated as *emrA*-like/*emrB*-like transporter systems in the genome sequence of *A. baumannii* ATCC 17978 strain were identified. These *emrA*-like/*emrB*-like gene pairs are A1S_0774/A1S_0775, A1S_0908/A1S_0909, A1S_1773/A1S_1772, and A1S_1800 (or A1S_1801)/A1S_1799. Similar analogs for these four gene pairs can also be found in *A. baumannii* ATCC 19606 and AYE strains (Supplementary data Table S1). To further characterize these four pairs, a sequence alignment was performed, and the results showed that the A1S_1773/A1S_1772 pair had the highest similarity with the Emr counterpart in *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Supplementary data Table S2 and Fig. S2). The *A. baumannii* A1S_1772 gene encoding a protein of 490 amino acids that shared 56.8% similarity with *E. coli* EmrB, 56.1% similarity with *K. pneumoniae* EmrB and 55.1% similarity with *P. aeruginosa* EmrB. In addition, the A1S_1772/EmrB protein contains five of the conserved residues within the drug extrusion consensus sequence (GPI LGPVLGG) that is usually detected in the MF family of proteins (Colmer *et al.*, 1998). On the other hand, the *A. baumannii* A1S_1773 gene, encoding a protein with 251 amino acids, contained a signature of the MFP proteins with two conserved domains: HlyD_3 and Biotin_lipoyl_2. Sequence alignments of *A. baumannii* A1S_1773 with EmrA homologs in other bacteria showed that A1S_1773 shared 47.4% similarity with *E. coli* EmrA, 50.3% similarity with *K. pneumoniae* EmrA and 50.8% similarity with *P. aeruginosa* EmrA. Therefore, A1S_1772 and A1S_1773 were chosen as a representative of EmrAB system and designated as *A. baumannii* EmrB and EmrA in the subsequent study.

To test if the *A. baumannii* EmrAB system is involved in the cell response to environment change, the expression of the *emrB*-like genes were determined by qRT-PCR. As shown in Fig. 1, expression of A1S_0775, A1S_0909, and A1S_1772 were 1.7-, 1.3-, and 2.2-folds higher in cells undergoing 20% sucrose osmotic stress than the control (without sucrose added), respectively.

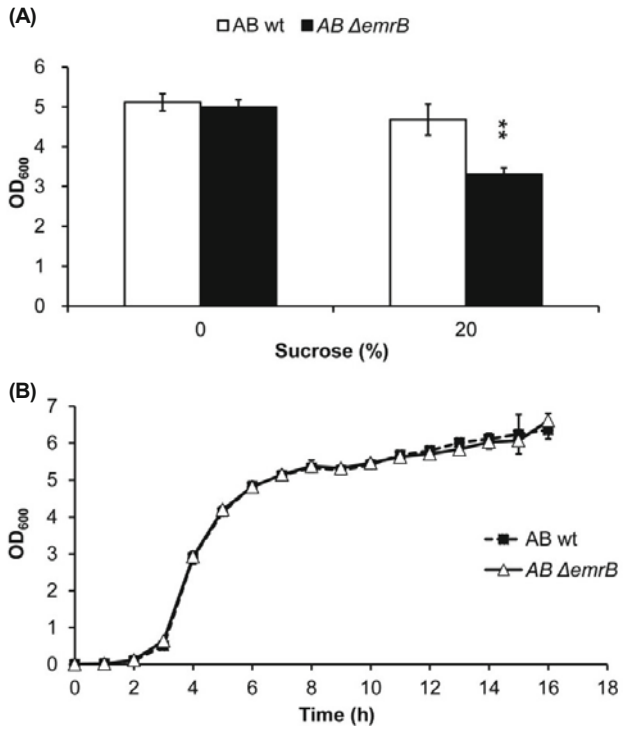

Fig. 2. Comparison of cell growth between the wild type and *emrB* deletion mutant. (A) The growth kinetics of the wild type and *emrB* deletion mutant were assessed in LB broth with or without 20% sucrose. The results were from three independent experiments. ** $P < 0.01$. (B) The growth curve was depicted with photometry (OD_{600}) performed on overnight cultures (37°C, 220 rpm, 16 h) in LB broth.

Co-transcription of the *emrA* and *emrB* genes

Although EmrA/EmrB has been characterized in *E. coli*, the biological functions of EmrA/EmrB in *A. baumannii* have not been explored. Genome analysis of *A. baumannii* ATCC 17978 showed that the coding sequences of *emrB* (A1S_1772) and *emrA* (A1S_1773) were located next to each other, suggesting that these two genes may be co-transcribed as an operon. To test this hypothesis, RT-PCR was performed (Supplementary data Fig. S3A). The result indicated that a 1,241-bp DNA fragment covering the junction between the *emrB* and *emrA* genes was obtained. Therefore, these two genes were co-transcribed as a single operon in *A. baumannii* ATCC 17978.

Construction of the *emrB* gene deletion (AB Δ*emrB*) mutant

To further study the role of the EmrAB pump in *A. baumannii*, in-frame *emrB* gene deletion mutants were generated. The successful construction of the AB Δ*emrB* mutant was demonstrated in Supplementary data Fig. S3B. The lack of the *emrB* (A1S_1772) gene in the genome was shown by PCR, and no transcript was detected by RT-PCR.

Comparison of the osmotic stress response and cell growth between the wild type and the AB Δ*emrB* mutant

To test whether A1S_1772 associates with an Emr efflux pump, the activity was measured by examining the exclusion of Hoechst (H) 33342 in the wild and the Δ*emrB* mutant (Richmond *et al.*, 2013). The ability of pumping H33342 out of the cells was compromised (13.5% increase of H33342 in the cells) after deletion of *emrB* gene (Supplementary data Fig. S4). This result might suggest that A1S_1772 function as an efflux pump like Emr transporters in other bacterial species.

As shown in Fig. 2A, the AB Δ*emrB* mutant showed growth retardation (30% decrease) under the condition of 20% sucrose concentration. However, no growth difference was perceived between the wild type strain and its *emrB* deletion mutant without the addition of 20% sucrose (Fig. 2B). These results indicated that the *A. baumannii* EmrAB pump system

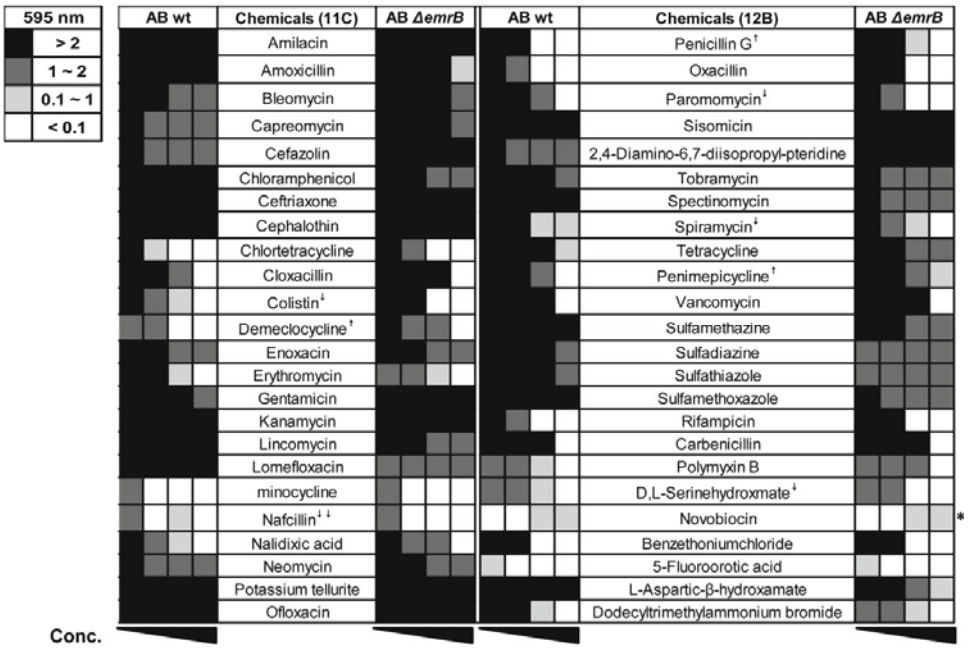

Fig. 3. Phenotype microarray assay between the wild type and *emrB* deletion mutant. Differential susceptibility to some of the studied 48 compounds was observed. Four different concentrations of each compound were placed in individual wells next to each other in a row with increasing concentrations from the left to the right. The growth of *A. baumannii* was determined by measuring the optical density at 595 nm (OD_{595}). All of the compounds more effective in inhibiting the AB Δ*emrB* mutant cell growth more were marked with ↓. In contrast, the compounds with which the AB Δ*emrB* mutant exhibited better resistance than wild type were marked with ↑. * The colour of the chemical compounds somehow interfered with observation of the bacterial growth.

may be required for normal bacterial growth under osmotic stress.

Comparison of antimicrobial susceptibility between the wild type and the AB *ΔemrB* mutant with a phenotypic microarray assay

Susceptibility to 48 compounds and antibiotics was compared between the wild type and the AB *ΔemrB* mutant (Fig. 3). After cell incubation in the PM11C microplate for 24 h, no AB Δ*emrB* cell growth was observed at low concentrations of colistin and nafcillin. Similarly, in the PM12B microplate there was no AB Δ*emrB* cell growth observed at low concentrations of paromomycin, spiramycin, and D,L-serine hydroxmate. However, AB Δ*emrB* was less susceptible to demeclocycline, penicillin G, and penimepicycline compared with the wild type strain.

MIC determination, spot assay, and time kill assay

Because colistin is one of the last resort antibiotics to treat the patients with multidrug resistant *A. baumannii* (MDRAB) infections, it is important to find out whether *emrB* gene (A1S_1772) deletion will lead to a increase in the colistin susceptibility of cells. The MIC of colistin for the wild type and AB *ΔemrB* mutant was 2 and 1 μg/ml, respectively. In the spot assay (Fig. 4), we did not observe growth of a 10^1 AB *ΔemrB* bacterial solution on an LB plate containing 0.5 μg/ml colistin, whereas 0.75 μg/ml colistin fully inhibited 10^4 AB *ΔemrB* cells. In the presence of 1 μg/ml colistin, no diluted bacterial solutions exhibited growth, except 10^5 of the AB wild type cells, which exhibited slight growth. All of the above findings further support the possible contribution of the EmrAB pump to colistin susceptibility in *A. baumannii*.

There were no differences in the surviving colony forming units (CFUs) between the wild type and *ΔemrB* mutant when colistin was not added to the LB agar (Fig. 5A). In the presence of 0.5 μg/ml colistin, these two tested strains had similar surviving CFU curves (Fig. 5B). However, when colistin concentration was increased to 1 μg/ml, the *ΔemrB* mutant showed a greater reduction in CFUs than the wild-type strain throughout the assay period with the lowest value being observed at 12 h (e.g., 3.1-$\log_{10}$ reduction at 12 h), which was followed by partial regrowth (Fig. 5C).

Expression of *emrB*-like genes in colistin resistance-induced *A. baumannii*

To further confirm the role of the *emrB* genes in colistin

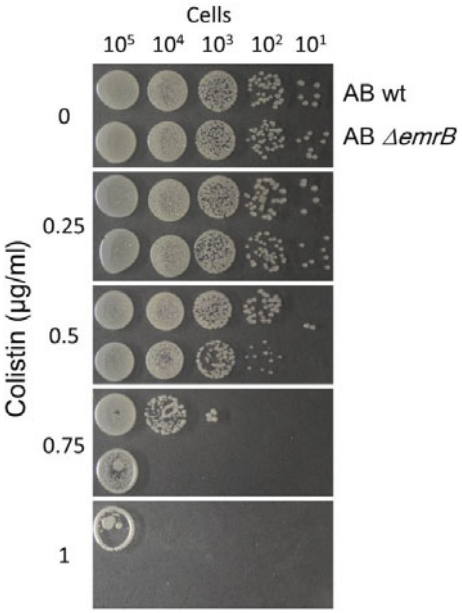

Fig. 4. Spot assay with colistin between the wild type and *emrB* deletion mutant. The bacteria were spotted (10 μl per spot) onto the LB agar plates containing different concentrations of colistin and were incubated at 37°C for 24 h. The wild-type strain exhibited better resistance to colistin than the *emrB* deletion mutant.

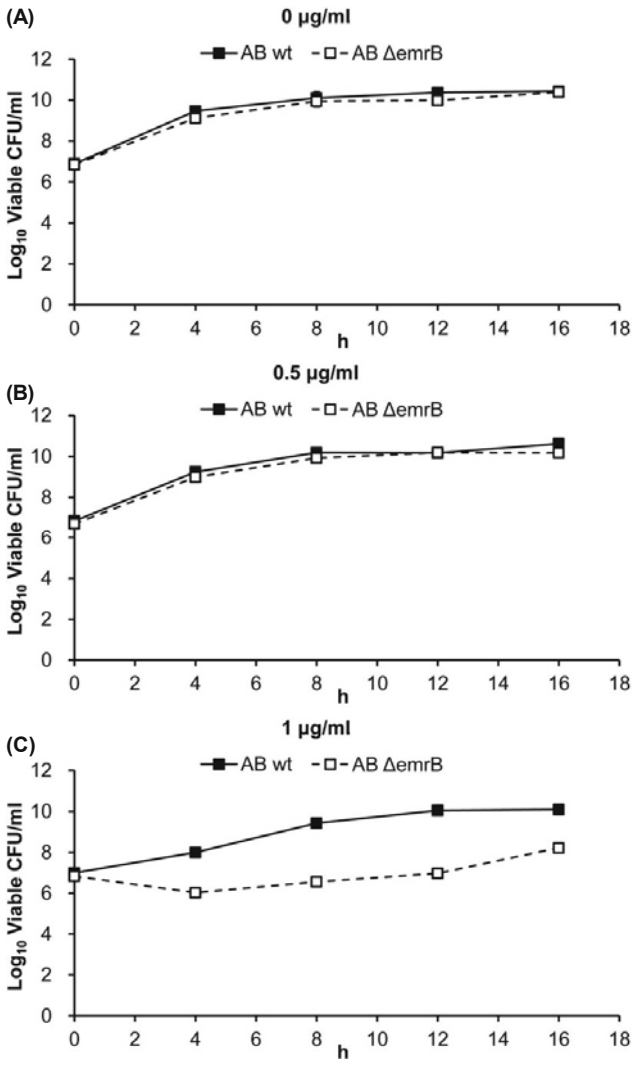

Fig. 5. Time-kill assays for the wild type and *emrB* deletion mutant without (A) or with 0.5 μg/ml (B) and 1 μg/ml (C) colistin. There were no differences in the CFUs between these two tested strains when no colistin or 0.5 μg/ml colistin was added to the LB agar. In the presence of 1 μg/ml colistin, the *emrB* deletion mutant showed a greater reduction in CFUs than the wild-type strain throughout the assay period with the lowest value being observed at 12 h.

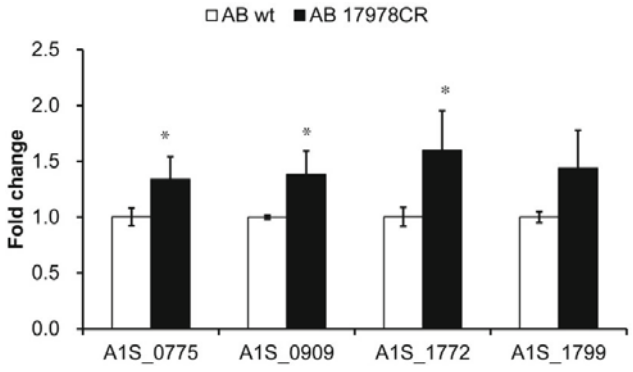

Fig. 6. Comparison of *emrB*-like gene expression in *A. baumannii* after colistin induction. The expression of the *emrB*-like gene between the wild type and colistin resistance-induced strains (AB 17978CR) was compared by qRT-PCR. The results were from three independent experiments. * $P < 0.05$.

susceptibility of *A. baumannii*, we compared *emrB*-like gene expression between the wild type and colistin resistance-induced strains (AB 17978CR) by qRT-PCR. The colistin resistance-induced strain was induced from *A. baumannii* ATCC 17978 strain. LB broth containing incremental colistin concentration (MIC on day 1, 2 × MIC on day 5, 4 × MIC on day 9) was inoculated with *A. baumannii* strain by serial passaging as previously described (Lin *et al.*, 2014) and the cultures were incubated at 37°C with shaking (220 rpm). The induced colistin-resistant strain was maintained in LB broth with 4 × MIC of colistin. The gene expression of A1S_0775, A1S_0909, A1S_1772, and A1S_1799 in the colistin resistance-induced *A. baumannii* was 1.3, 1.4, 1.6, and 1.4-fold higher than in the wild type strain, respectively (Fig. 6). These data further imply that there is a contribution of the EmrAB pump system to colistin susceptibility in *A. baumannii*.

Discussion

Since Lomovskaya had first described the gene cloning of *emrA* and *emrB* and their relationship with multidrug resistance in *E. coli* (Lomovskaya and Lewis, 1992), there is still few studies about EmrAB efflux pump system in bacteria. Of the few studies, *E. coli* strain CDM5 was found through activating *emrB* as the mechanism for thiolactomycin resistance (Furukawa *et al.*, 1993), whereas the fluoroquinolone-resistant mutants of *Salmonella enterica* Serovar Typhimurium had substantial increased *emrB* gene expression (Chen *et al.*, 2007). However, the contribution of EmrAB pump to antibiotic resistance in other bacteria including *A. baumannii* is mostly unknown.

In this study, A1S_1773 and A1S_1772 were demonstrated as *A. baumannii* EmrA and EmrB homolog by silicon analysis, expression analysis and efflux assay. We showed that this putative efflux pump system provides *A. baumannii* with better ability to resist osmotic stress and several chemical compounds, including colistin. MDRAB has emerged as an important pathogen of nosocomial infections (Lin and Lan, 2014). As colistin is one of the last resort antibiotics to treat MDRAB, the emergence of colistin resistance is worrisome. Efflux pumps action is usually underestimated as a cause of antibiotic resistance. However, the evidence is that bacteria possess a variety of efflux systems able to extrude in more or less efficient manner antibiotics and disinfectants (Hooper, 2005). Our result disclosed decrease of colistin susceptibility in the AB *ΔemrB* mutant. This finding suggests the possible role of certain efflux pump systems in contributing to colistin resistance of *A. baumannii*.

The antibacterial effect of colistin on Gram-negative bacteria acts through a two-step mechanism (Bialvaei and Samadi Kafil, 2015). Colistin binds to lipopolysaccharide (LPS) while displacing cations from the membrane, leading to disruption of the outer membrane and to the loss of cellular contents, thus killing the bacterium. However, the mechanism of action for colistin, which is mainly through exerting its effect extracellularly, raises an issue about how EmrAB pumps out the anbibiotics. RosA/RosB, a temperature-regulated efflux pump/potassium antiporter system, can mediate resistance to cationic antimicrobial peptides (CAMPs), such as polymyxin B, in *Yersinia* (Bengoechea and Skurnik, 2000). This system is induced at a higher temperature (37°C) and it becomes a critical mechanism of CAMPs resistance by pumping out the CAMPs and by inducing the acidification of the cytoplasm. The lower intracellular pH then can act as a positive regulatory signal for the induction of other CAMPs resistance mechanisms that might involve changes at the outer membrane level. It is unclear whether EmrAB can work as mediators of colistin resistance similar to that of RosA/RosB in *Yersinia*.

To date, there are two main mechanisms responsible for colistin resistance of *A. baumannii*. One mechanism is the complete loss of LPS resulting from mutation or insertion in *lpxA*, *lpxC*, or *lpxD* (Moffatt *et al.*, 2010, 2011). Another mechanism is the addition of phosphoethanolamine to LPS, which is mediated through *pmrAB* gene mutations (Adams *et al.*, 2009). Loss or decreased OmpW porin was also reported as a possible colistin resistance mechanism in *A. baumannii* mutant (Lee *et al.*, 2011). We found increased OmpW (A1S_0292) gene expression in our colistin-resistant strain (data not shown). The whole picture of colistin resistance in *A. baumannii* and how these pump systems interact and exert their effect, including OmpW and EmrAB, deserved further studies.

This study showed that the *emrB* gene (A1S_1772) deletion mutant has pleiotropic effects on cell physiology and may affect the intrinsic susceptibility of bacteria towards colistin, other antibiotics and environmental conditions. Together, this study suggests a possible contribution of the EmrAB system to colistin susceptibility in *A. baumannii*, which can complement the known mechanism and may help in the battle against this superbug.

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