Journal of Microbiology, Immunology and Infection (2017) 50, 224–231

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

Distribution of different efflux pump genes in clinical isolates of multidrug-resistant *Acinetobacter baumannii* and their correlation with antimicrobial resistance

Ming-Feng Lin [a,b], Yun-You Lin [b], Chi-Chao Tu [c], Chung-Yu Lan [b,d,*]

[a] *Department of Medicine, National Taiwan University Hospital Chu-Tung Branch, Hsin-Chu County, Taiwan*
[b] *Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsin-Chu City, Taiwan*
[c] *Department of Laboratory Medicine, Keelung Hospital, Minister of Health and Welfare, Keelung, Taiwan*
[d] *Department of Life Science, National Tsing Hua University, Hsin-Chu City, Taiwan*

Received 25 November 2014; received in revised form 4 February 2015; accepted 18 April 2015
Available online 14 May 2015

KEYWORDS
Acinetobacter baumannii;
antibiotic resistance;
efflux pumps

Abstract *Background/purpose:* Efflux pumps are one of the major mechanisms of antimicrobial resistance in *Acinetobacter baumannii*. This study aimed to understand the distribution of different types of pump genes in clinical isolates of multidrug-resistant *A. baumannii* (MDRAB) and to reveal the relationship between their presence and expression with antimicrobial resistance.
Methods: MDRAB isolates were collected from five hospitals in Taiwan. Different categories of pump genes, including *adeB*, *adeJ*, *macB*, *abeM*, *abeS*, *emrA*-like, *emrB*-like, and *craA*, were chosen, and their presence in the collected isolates was determined. Three induced resistant strains of *A. baumannii* ATCC 17978 to tigecycline, imipenem, and amikacin were also included. The expressions of the selected pump genes were determined using quantitative reverse transcription-polymerase chain reaction.
Results: Twenty-one MDRAB clinical isolates were obtained from five hospitals. All of the studied pump genes were present in the collected MDRAB isolates except one isolate that lacked the *emrA*-like gene. The gene expression of these efflux pumps was variable among the strains. The upregulation of the *adeB*, *adeJ*, and *macB* genes was responsible for tigecycline resistance, and the increased *abeS* expression was strongly related to amikacin resistance. Of all

* Corresponding author. Institute of Molecular and Cellular Biology, National Tsing Hua University, Number 101, Section 2, Kuang-Fu Road, Hsinchu 30013, Taiwan.
E-mail address: cylan@life.nthu.edu.tw (C.-Y. Lan).

http://dx.doi.org/10.1016/j.jmii.2015.04.004

the antibiotics studied, tigecycline was the strongest inducer of gene expression for many efflux pumps in *A. baumannii*.
Conclusion: Efflux pump genes are universally present in the collected clinical MDRAB isolates. The upregulation of the *adeB*, *adeJ*, *macB* and *abeS* genes is more related with antibiotic resistance.

Introduction

Acinetobacter baumannii has evolved from being a commensal bacteria in health care facilities to constitute one of the most important microorganisms responsible for nosocomial infections and hospital outbreaks in recent decades.[1] The reasons for the success of *A. baumannii* may be related to the potential for this organism to achieve dynamic reorganization and rapid evolution of its genome, including the acquisition and expression of exogenous antibiotic resistance genes under environmental selective pressure.[2] A global surveillance study of *A. baumannii* noted significant increases in antimicrobial resistance worldwide from 2004 to 2009.[3] The isolates collected from Asia and the Pacific Rim in a previous study demonstrated increases in resistance for all antimicrobials, ranging from a 19.1% increase in ceftazidime resistance to a 38.9% increase in levofloxacin resistance. The imipenem (IPM) resistance rate of *A. baumannii* from another worldwide collection between 2005 and 2009 reached resistance rates $>$ 50%.[4]

The major mechanisms known to confer resistance to different class of antibiotics in *A. baumannii* include β-lactamases, aminoglycoside-modifying enzymes, permeability defects, multidrug efflux pumps, and alteration of target sites.[5,6] Among these mechanisms, the role of bacterial efflux pumps in multidrug resistance has been emphasized.[7,8] Antimicrobials are excreted out of the cell, which leads to a reduction in drug accumulation and an increase in minimum inhibitory concentrations (MICs).[9] Each efflux pump consists of three components: the inner membrane transporter, the outer membrane channel, and the periplasmic lipoprotein.[10] Multidrug efflux pumps are generally chromosome-encoded, and their expressions often result from mutations in regulatory genes. However, drug-specific efflux pumps are encoded by mobile genetic elements whose acquisition is sufficient for resistance.[11] Four categories of efflux pumps, including the resistance—nodulation—division (RND) family, major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, and small multidrug resistance (SMR) family of transporters, have been linked to *A. baumannii* antimicrobial resistance.[9] Of these different pumps, the RND and MFS families of transporters have been the most studied. AdeABC, a RND-type efflux pump, is not only associated with aminoglycoside resistance,[12] but is also involved in the level of susceptibility to many antibiotics, including tigecycline (TGC).[13] Overexpression of other RND-type efflux pumps, including AdeFGH and AdeIJK, contributes to multidrug resistance in *A. baumannii*.[14,15] A number of MFS efflux pumps, including TetA,[16] CmlA,[8] CraA,[17] and AmvA,[18] mediate resistance to different types of antibiotics. A mutant of AbeS, an SMR efflux pump, exhibits erythromycin and chloramphenicol resistance in *A. baumannii*,[19] whereas a MATE family pump, AbeM, was reported to confer resistance to fluoroquinolones and IPM.[20]

Fournier et al[21] have described 46 open reading frames (ORFs) that are putatively associated with resistance to antimicrobials outside an 86-kb gene resistance island using a comparative genomic approach. Of these 46 ORFs, 32 ORFs were associated with the RND family, seven with the MFS, two with the MATE family, and one with the SMR family. Although these previous studies have revealed the contribution of different categories of efflux pumps to antimicrobial resistance in *A. baumannii*, the role of some other efflux pumps, such as MacAB-tolC and EmrAB, which have been well described in other species of bacteria,[22,23] have yet to be explored. Because the choice of antimicrobial treatment for multidrug resistant *A. baumannii* (MDRAB) is severely limited, there are only few effective options available, including polymyxins and TCGs.[5] Therefore, this study aimed to understand the distribution of different types of pump genes in clinical isolates of *A. baumannii* and to reveal the relationship between their presence and expression with antimicrobial resistance to provide a basis for developing new targets for future therapies.

Methods

Specimen collection and strain identification

MDRAB isolates were collected from five hospitals in Taiwan with five isolates from the Hsin-Chu branch of National Taiwan University Hospital (NTUH), four from the Chut-Tung branch of NTUH, seven from the Hsin-Chu branch of Taipei Veterans General Hospital, four from Catholic Mercy Medical Foundation, and one from Hualien Tzu Chi Hospital. Each isolate was sampled from a different patient and classified as genus *Acinetobacter* using the Vitek system (Biomerieux Vitek, Inc., Hazelwood, MO, USA). *A. baumannii* was further identified by one-tube multiplex polymerase chain reaction (PCR) based on the method of Chen et al.[24]

Induction of resistance for amikacin, IPM, or TGC

The induced TGC-resistant *A. baumannii* strain was from our previous study,[25] whereas the induced amikacin (AMK)-

resistant and IPM-resistant *A. baumannii* strains were from a study by Kuo et al.[26] Serial passaging was performed as previously described by Li at al[27] with several modifications.

Antimicrobial susceptibility tests

Susceptibilities to antimicrobial agents were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute.[28] The tested agents included ampicillin/sulbactam (SAM), piperacillin/tazobactam, cefazolin, cefmetazole, cefotaxime, ceftazidime, cefepime (FEP), IPM, meropenem (MEM), AMK, gentamicin, ciprofloxacin, levofloxacin, trimethoprim/sulfamethoxazole, and TGC.

Detection of different efflux pump genes in clinical isolates of MDRAB

Different categories of pump genes were chosen to determine their presence in the collected clinical isolates of MDRAB to understand their distribution, including RND pump genes *adeJ* and *adeB*, MFS pump genes *craA* and *emrB*-like (A1S_1772), membrane fusion protein gene *emrA*-like (A1S_1800), MATE group *abeM*, SMR group *abeS*, and macrolide ABC transporter gene *macB* (A1S_0536). The primers used for detection of the selected genes are listed in Table 1.

DNA manipulation

Plasmid DNA was prepared with the FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen, Ping-Tung, Taiwan). *A. baumannii* genomic DNA was extracted as described previously.[29] Briefly, PCR amplification was performed in a Thermo Hybaid PXE 0.2 HBPX02 Thermal Cycler (Thermo Scientific, Redwood, CA, USA), using Pro*Taq* DNA Polymerase (Protech, Taipei, Taiwan) or the KAPA HiFi PCR Kit (Kapa Biosystems, Boston, MA, USA). DNA fragments were extracted from agarose gels and purified using the GeneKlean Gel Recovery & PCR CleanUp Kit (MDBio, Inc., Taipei, Taiwan). Nucleotide sequences of the PCR products were verified using an ABI 3730XL DNA Analyzer (Applied Biosystems, South San Francisco, CA, USA).

Table 1 Oligonucleotides used in this study.

Primer name	Sequence (5′ to 3′)
q*adeB*_F	ACAAGACCGCGCTAACTTAGGT
q*adeB*_R	TGCCATTGCCATAAGTTCATCT
q*adeJ*_F	AGCTGGTGCTATGGGCGTTA
q*adeJ*_R	GCCACCCCATGCAATACG
q*macB*_F	CGGAATGGGTTCGGATGAC
q*macB*_R	CGGCTCATGACCGTGGTATAA
q*emrB*_F	GCGGGATGATTCCGACTTC
q*emrB*_R	TGAGCGTTTTGGTTCTGGAAA
q*emrA*_F	AACAGAGGCCCAGCTTAGAAAA
q*emrA*_R	AGCGTGCGGTATTATCTTTAGTGA
q*abeS*_F	TGTGGGTTATGCAGTTGCTTTT
q*abeS*_R	GGCATAGGCAATCCCGATT
q*abeM*_F	TGGGTATGCCCGCTGTAAC
q*abeM*_R	ATGGCCTAATGCTTCGGAATAG
q*craA*_F	CCTGATTCAGCCAGCCATGT
q*craA*_R	GAAGACGGCGCCCAAGT
q16s rRNA_F	AGCATTTCGGATGGGAACTTTA
q16s rRNA_R	GTCGTCCCCGCCTTCCT

RNA isolation, reverse transcription-PCR, and quantitative reverse transcription-PCR

RNA isolation, reverse transcription (RT)-PCR and quantitative RT (qRT)-PCR methods were performed as described in our previous study.[25] The cDNAs were used in PCR reactions with different primers for qRT-PCR (Table 1), which was carried out with a StepOne Real-Time PCR System (Life Technologies, Grand Island, NY, USA). Briefly, each 20-μL reaction mixture contained 25 ng cDNA, 10 μL Power SYBR green PCR master mix (Life Technologies), and 300nM each forward and reverse primer. The reactions were performed with one cycle at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The 16S rRNA transcript was used as an endogenous control for qRT-PCR. The data were analyzed using the StepOne version 2.1 software (Life Technologies).

Statistical analysis

The susceptibility difference was analyzed using Student *t* test. The differences between two groups of isolates were considered significant at $p < 0.05$. Data entry and analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Antimicrobial susceptibility of MDRAB isolates and induced resistant strains

All of the 21 *A. baumannii* clinical isolates obtained from the five hospitals are multidrug-resistant and showed 100% nonsusceptibility to many of the tested antimicrobial agents, except SAM, FEP, IPM, MEM, AMK, trimethoprim/sulfamethoxazole, and TGC (Table 2). Two isolates, VGH2 and VGH7, showed pan-drug resistance, whereas the CT11 and CT13 isolates had more antimicrobial susceptibility than the others. Both AMK and TGC were the most effective antimicrobials against the collected isolates (71.4% susceptible, 15/21). Of the collected isolates, 19.0% (4/21) were susceptible to trimethoprim/sulfamethoxazole, and only 9.5% (2/21) were susceptible to SAM, FEP, IPM, or MEM. The antibiograms of VGH1 and VGH2 were similar to those of the amikacin-induced strain (ABamk) and imipenem-induced strain (ABipm), respectively. VGH7 had the highest TGC MIC ($\geq$ 8 μg/mL). Therefore, the VGH1, VGH2, VGH7, HC4, CT11, and CT13 strains along with the wild type, induced and ABhl1 strains were chosen for analysis of pump gene expression by qRT-PCR.

Table 2 Antimicrobial susceptibility of the collected MDRAB isolates and induced-resistant strains.

Strains	SAM	TZP	CFZ	CMZ	CTX	CAZ	FEP	IPM	MEM	AMK	GEN	CIP	LVX	SXT	TGC
ABwt	S (≤ 2)	S (≤ 4)	R (≥ 64)	R (32)	S (8)	S (2)	S (≤ 1)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 2)	S (≤ 1)	S (≤ 0.25)	S (≤ 0.12)	S (40)	S (≤ 2)
ABtc	S (≤ 2)	S (≤ 4)	R (≥ 64)	R (≥ 64)	S (8)	S (4)	S (4)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 2)	S (4)	I (2)	S (1)	R (160)	R (≥ 8)
ABipm	S (8)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	I (8)	R (≥ 16)	R (≥ 64)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	I (4)
ABamk	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
ABhl1	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	R (≥ 8)
VGH1	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
VGH2	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	I (8)	R (≥ 16)	R (≥ 64)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	R (8)
VGH3	I (16)	R (≥ 128	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	I (8)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	R (8)
VGH4	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	R (8)
VGH5	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
VGH6	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (8)	R (≥ 16)	R (≥ 4)	R (≥ 8)	S (≤ 20)	R (≥ 8)
VGH7	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 16)	R (≥ 16)	R (≥ 64)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	R (≥ 8)
HC1	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	I (4)	S (≤ 20)	S (≤ 2)
HC2	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (160)	S (≤ 2)
HC3	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 16)	R (≥ 16)	R (≥ 64)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
HC4	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	R (≥ 64)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
HC5	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 16)	R (≥ 16)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (160)	S (≤ 2)
CT11	S (4)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	I (16)	S (1)	S (0.5)	S (4)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (160)	S (≤ 2)
CT12	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	R (8)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (160)	S (≤ 2)
CT13	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	S (1)	S (1)	S (≤ 2)	S (≤ 1)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
CT14	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (8)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (160)	S (≤ 2)
CM1	R (≥ 32)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (≤ 2)	S (≤ 1)	R (≥ 4)	R (≥ 8)	S (≤ 20)	S (≤ 2)
CM2	S (4)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	I (16)	R (≥ 16)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
CM3	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 16)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	R (≥ 320)	S (≤ 2)
CM4	I (16)	R (≥ 128)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 16)	R (≥ 16)	S (≤ 2)	R (≥ 16)	R (≥ 4)	R (≥ 8)	S (≤ 20)	S (≤ 2)

ABamk = amikacin-induced ABwt; ABhl1 = an *Acinetobacter baumannii* strain from Hualien Tzu Chi Hospital; ABipm = imipenem-induced ABwt; ABtc = induced tigecycline-resistant ABwt; ABwt = *A. baumannii* ATCC 17978; AMK = amikacin; CAZ = ceftazidime; CFZ = cefazolin; CIP = ciprofloxacin; CM = Catholic Mercy Hospital; CMZ = cefmetazole; CT = Chut-Tung branch of National Taiwan University Hospital; CTX = cefotaxime; FEP = cefepime; HC = Hsin-Chu branch of National Taiwan University Hospital; I = intermediate; IPM = imipenem; GEN = gentamicin; LVX = levofloxacin; MEM = meropenem; R = resistant; S = susceptible; SAM = ampicillin/sulbactam; SXT = trimethoprim/sulfamethoxazole; TGC = tigecycline; TZP = piperacillin/tazobactam; VGH = Hsin-chu branch of Taipei Veterans General Hospital.

Distribution of different pump genes in the clinical isolates of MDRAB

Based on the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/gene/?term=A1S_0538), A1S_0535 (*tolC*), A1S_0536 (*macB*), A1S_0537, and A1S_0538 (*macA*) are counterparts of the *macAB_tolC* gene in *A. baumannii* ATCC 17978. The *emrB*-like gene A1S_1772 and gene A1S_1773 were shown to function as an operon by RT-PCR along with A1S_1799 and *emrA*-like gene A1S_1800 (data not shown). RND pumps *adeJ* and *adeB*, MFS pump *craA* and *emrB*-like (A1S_1772) genes, MATE group *abeM*, SMR group *abeS*, and macrolide ABC transporter gene *macB* (A1S_0536) were all present in the 24 clinical isolates of MDRAB, the wild-type strain, and their induced resistant strains. Membrane fusion protein gene *emrA*-like (A1S_1800) was also present in all of the studied isolates except one from the Chu-Tung branch of the NTUH (CT3).

Relative expression of efflux pump genes in MDRAB

In all of the studied MDRAB strains, including three antibiotic-induced strains and seven clinical isolates, *adeB*, *abeS*, and *abeM* gene expressions all increased compared with that of *A. baumannii* ATCC 17978, although the increase in *adeB* gene expression in ABtc, VGH1, and HC4 and *abeM* gene expression in ABhl1 were not statistically significant (Table 3). By contrast, the relative expression of *emrA*-like gene A1S_1800 and *emrB*-like gene A1S_1772 decreased in all of the studied MDRAB strains except ABtc, which still showed increased expression of these two genes. However, the relative gene expression of *adeJ*, *macB*, and *craA* varied among the studied MDRAB strains. ABtc, ABipm, ABhl1, and VGH7 had increased *adeJ* gene expression, whereas HC4 and CT11 showed decreased relative gene expression of *adeJ*. Six strains—ABtc, ABipm, ABamk, ABhl1, VGH7, and CT11—had increased expression of *macB*, but CT13 showed decreased expression. The decreased relative expression of *craA* could be observed in ABhl1, HC4, and CT13, and ABtc was the only strain with significantly increased *craA* gene expression.

Relationship of the expression of efflux pump genes and antimicrobial resistance

Because the collected MDRAB were resistant to most of the tested antibiotics, especially β-lactam antibiotics and fluoroquinolones, only the antimicrobial susceptibility of AMK and TGC were chosen for further analysis. To reveal the relationship between efflux pump genes and their expression, each antibiotic category was divided into two groups with MIC values of $\geq$ 64 μg/mL and $\geq$ 8 μg/mL for AMK (Figure 1) and TGC (Figure 2), respectively. The strains with TGC MIC $\geq$ 8 μg/mL had more gene expression of *adeB* ($p = 0.059$), *adeJ* ($p = 0.023$), and *macB* ($p = 0.033$) than the susceptible strains, whereas statistically significantly increased expression of the *abeS* gene was observed in the AMK-resistant group ($p = 0.008$).

Table 3 Relative expression[a] of efflux pump genes in some collected MDRAB isolates and induced resistant strains.

Strains	*adeB*	*adeJ*	*macB*	*abeS*	*emrA*[b]	*emrB*[c]	*abeM*	*craA*
ABwt	1.05 ± 0.35	1.00 ± 0.11	1.01 ± 0.20	1.00 ± 0.11	1.01 ± 0.14	1.00 ± 0.10	1.02 ± 0.22	1.02 ± 0.28
ABtc	329.09 ± 86.00*	9.29 ± 1.91*	12.26 ± 0.67***	0.65 ± 0.06*	1.96 ± 0.08**	4.30 ± 0.44**	2.01 ± 0.28**	2.25 ± 0.13**
ABipm	58.22 ± 22.22*	1.74 ± 0.06**	2.14 ± 0.36*	16.53 ± 2.69**	0.60 ± 0.07*	0.39 ± 0.07**	3.52 ± 0.71*	1.62 ± 0.25
ABamk	22.92 ± 17.65	1.21 ± 0.20	2.39 ± 0.45*	16.43 ± 1.95**	0.33 ± 0.03*	0.97 ± 0.07	1.70 ± 0.11*	1.40 ± 0.25
ABhl1	98.94 ± 25.47*	4.73 ± 0.88*	2.91 ± 0.72*	6.52 ± 1.18*	0.68 ± 0.18	0.45 ± 0.16**	1.26 ± 0.15	0.41 ± 0.11*
VGH1	10.18 ± 8.84	1.25 ± 0.16	1.18 ± 0.05	5.23 ± 1.26*	0.43 ± 0.29	0.48 ± 0.08**	2.15 ± 0.27**	0.78 ± 0.10
VGH2	36.07 ± 11.62*	1.03 ± 0.11	1.93 ± 0.45	15.76 ± 3.63*	0.30 ± 0.11**	0.19 ± 0.04**	2.96 ± 0.30**	1.41 ± 0.06
VGH7	47.50 ± 7.49*	3.01 ± 0.75*	6.24 ± 1.19*	17.24 ± 3.26*	0.34 ± 0.13**	0.21 ± 0.04**	4.46 ± 0.67**	0.99 ± 0.12
HC4	1.86 ± 1.03	0.55 ± 0.20*	1.54 ± 0.29	25.34 ± 2.13**	0.27 ± 0.15**	0.26 ± 0.13**	1.73 ± 0.29*	0.27 ± 0.08*
CT11	30.10 ± 8.71*	0.36 ± 0.19*	1.73 ± 0.13*	8.38 ± 2.49*	0.52 ± 0.12*	0.93 ± 0.20	4.07 ± 0.66**	1.18 ± 0.12
CT13	4.91 ± 1.63*	0.56 ± 0.27	0.42 ± 0.20*	12.42 ± 0.93**	0.00 ± 0.00**	0.31 ± 0.19*	5.42 ± 0.92*	0.36 ± 0.29*

[a] The numbers in the cells represent fold changes compared to those of ABwt.
[b] *emrA* represents A1S_1800.
[c] *emrB* represents A1S-1772.
ABamk = amikacin-induced ABwt; ABipm = imipenem-induced ABwt; ABhl1 = an *Acinetobacter baumannii* strain from Hualien Tzu Chi Hospital; ABtc = tigecycline-induced ABwt; ABwt = *A. baumannii* ATCC 17978; CT = Chut-Tung branch of National Taiwan University Hospital; HC = Hsin-Chu branch of National Taiwan University Hospital; VGH = Hsin-chu branch of Taipei Veterans General Hospital.
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

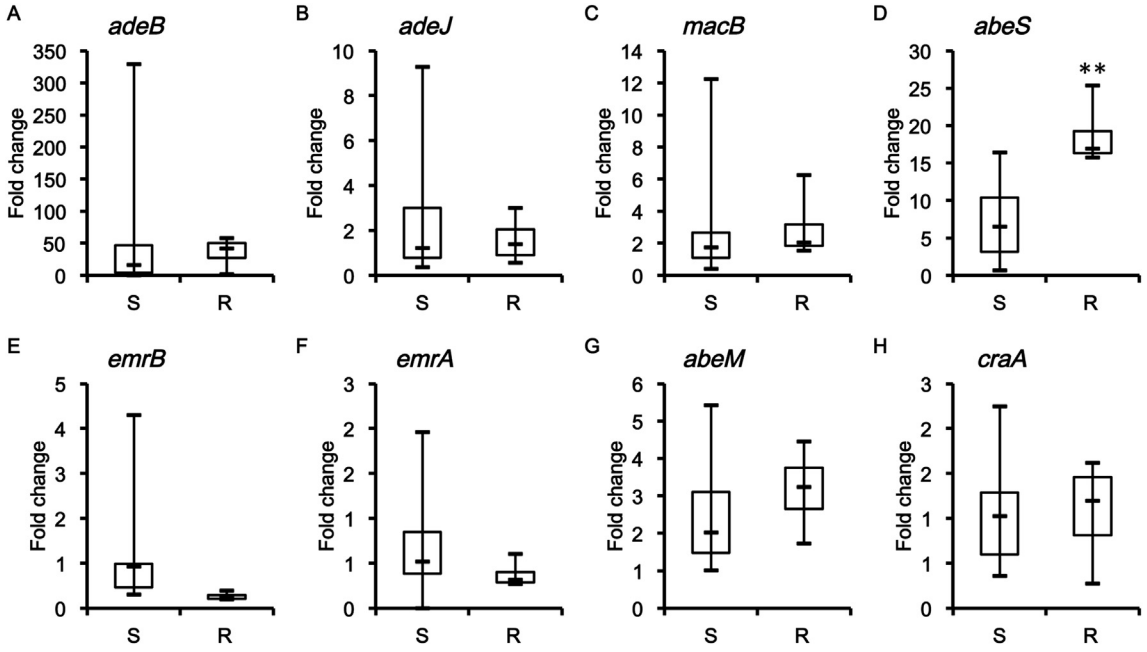

Figure 1. Relationship between pump gene expression and amikacin resistance. The clinical isolates were divided into two groups by minimum inhibitory concentration (MIC) values with a cutoff point of $\geq$ 64 μg/mL for amikacin. The strains with amikacin MICs $\geq$ 64 μg/mL had more gene expression of *abeS* than the more susceptible strains ($p = 0.008$).

Discussion

The role of efflux pumps in the antimicrobial resistance of *A. baumannii* is well reviewed in several previous reports.[8,9] The linear relationship between the log-transformed expression values of the AdeABC efflux pump genes and the log-transformed TGC MIC values provides solid evidence of the contribution of some efflux pumps to the resistance to certain antimicrobials.[30] The importance of efflux pumps in multidrug resistance in *A. baumannii* is

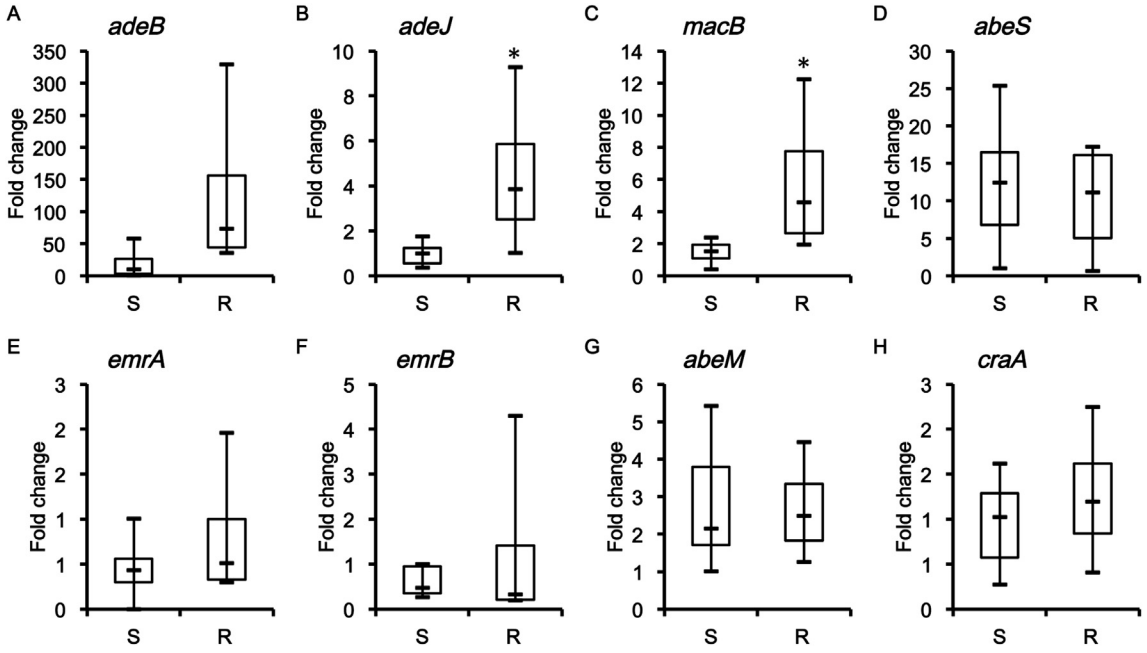

Figure 2. Relationship between pump gene expression and tigecycline resistance. The clinical isolates were divided into two groups by minimum inhibitory concentration (MIC) values with a cutoff point of $\geq$ 8 μg/mL for tigecycline. The strains with tigecycline MICs $\geq$ 8 μg/mL had more gene expression of *adeB* ($p = 0.059$), *adeJ* ($p = 0.023$), and *macB* ($p = 0.033$) than the more susceptible strains.

further supported by the fact that the presence of efflux pump inhibitors, such as 1-(1-naphthylmethyl)-piperazine,[31] phenyl-arginine-β-naphthylamide,[31] or carbonyl cyanide 3-chlorophenylhydrazone,[32] can reverse the resistance pattern. However, the previous studies of the relationship between individual efflux pumps and antimicrobial resistance were often undertaken in reference strains or a single clinical isolate. Few studies have dealt with differences in the phenotype of antimicrobial resistance and efflux pump gene expression among MDRAB clinical isolates from the different hospitals or sources. Our study showed the complexity of the relationship between pump gene expression and antimicrobial resistance in MDRAB. It is known that the mechanisms contributing to antimicrobial resistance of MDRAB, at least including β-lactamases, multidrug efflux pumps, aminoglycoside-modifying enzymes, and alteration of permeability, are complicated.[5,6] By analyzing the qRT-PCR results of this study, the efflux pumps only play a partial role on multidrug resistance of MDRAB. The resistance to one type of antibiotics often involves several relevant efflux pumps, if any, and one type of efflux pump system can contribute resistance to different classes of antibiotics simultaneously in *A. baumannii*. Because the efflux pump—for example, *AdeAB* in *A. baumannii* ATCC 17978—can recruit another outer membrane protein to form a functional tripartite complex,[8] the activities of the pump systems were evaluated mainly through measuring the transporter gene (e.g., *adeJ*, *macB*, *adeB*) expression, not porin gene (e.g., *adeK*, *tolC*, *adeC*) expression, by qRT-PCR in this study.

Currently, little is known about the epidemiology of various efflux pumps in *A. baumannii*. It was estimated that 53—97% of *A. baumannii* clinical strains harbor the *adeABC* operon,[8] but the presence of other pump genes have not been explored yet. The high prevalence rate of the different efflux pump genes in the clinical isolates of MDRAB in the present study suggested that MDRAB may be equipped with more machinery to adapt to its environment and survive. This notion is supported by the concept proposed by Piddock,[33] who thought that multidrug-resistance efflux pumps have roles in bacterial pathogenicity. However, the number of the clinical isolates in this study is limited, which may not be representative of the clinical isolates from the general population. Therefore, a large-scale study is necessary to verify our hypothesis in the future.

The emergence of MDRAB is worldwide. The Taiwan Surveillance of Antimicrobial Resistance program showed from 2002 to 2010 that the prevalence of the extensively drug-resistant *A. baumannii* complex (XDRABC) increased from 1.3% in 2002 to 41.0% in 2012.[34] One of the risk factors, acquiring XDRABC, is prior broad-spectrum antibiotic use. Because *A. baumannii* acquires resistance traits easily in the hospital environment with an abundance of antibiotics, we mimicked the same process in the laboratory through induction of the wild-type strains to enhance development of their resistance to TGC, IPM, and AMK. IPM and AMK are strong inducers of an antimicrobial resistance phenotype in *A. baumannii* with moderately upregulated expression of most pump genes, whereas TGC exposure induced TGC and trimethoprim/sulfamethoxazole resistance with markedly increased gene expression of *adeB*, *adeJ*, *macB*, and *emrB*. It is obvious that the emergence of antimicrobial resistance after induction by these three antibiotics is not all through upregulation of the efflux pump systems. However, by analyzing the antimicrobial susceptibility test and pump gene expression of ABtc, we can speculate that TGC is a strong inducer of gene expression for many drug transporters, but is not a potent inducer of multidrug resistance like IPM[26] in *A. baumannii*.

The main resistance mechanism for β-lactam antibiotics and fluoroquinolones are β-lactamases and DNA gyrase, respectively.[1] No definite relationship between the studied efflux pump gene expression and resistance to these two classes of antibiotics could be observed in the present study. According to the results in Table 3 and Figure 1, we assume that AdeABC, AdeIJK, and MacAB-TolC are important efflux pump systems that contribute to the antimicrobial resistance of certain antibiotics, especially TGC. Previous studies have shown that *abeS* and *abeM* can confer resistance to some antibiotics.[19,20] Our study also showed that both genes had upregulated gene expression in most of the tested strains. In addition, the AMK-resistant group had a statistically significant expression increase of the *abeS* gene. Although EmrAB pumps have been shown to be associated with resistance to certain antibiotics in *Escherichia coli*, and CraA was displaying strong substrate affinity toward chloramphenicol, it seems that they are not important pump systems that contribute antimicrobial resistance to MDRAB.

In conclusion, we found that the studied efflux pump genes are universally present in the collected clinical MDRAB strains, which had varied pump gene expression. The upregulation of *adeB*, *adeJ*, and *macB* genes are responsible for TGC resistance, and the increased *abeS* expression is strongly related with AMK resistance. Of all the antibiotics studied, TGC is the strongest inducer of gene expression for many efflux pumps in *A. baumannii*. All of these findings pave a path for the development of efflux pump inhibitors as adjuvant therapies in combating MDRAB.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by a grant from National Taiwan University Hospital, Chu-Tung Branch. The authors also thank Hsiu-Chou Yeh at the Hsin-Chu branch of National Taiwan University Hospital, Mei-Ju Yeh at Catholic Mercy Medical Foundation, and Bao-Ju Teng at the Hsin-Chu branch of Taipei Veterans General Hospital for providing the clinical *A. baumannii* strains.

References

1. Roca I, Espinal P, Vila-Farres X, Vila J. The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Front Microbiol* 2012;**3**:148.

2. Visca P, Seifert H, Towner KJ. *Acinetobacter* infection—an emerging threat to human health. *IUBMB Life* 2011;**63**:1048—54.
3. Morfin-Otero R, Dowzicky MJ. Changes in MIC within a global collection of *Acinetobacter baumannii* collected as part of the Tigecycline Evaluation and Surveillance Trial, 2004 to 2009. *Clin Ther* 2012;**34**:101—12.
4. Mendes RE, Farrell DJ, Sader HS, Jones RN. Comprehensive assessment of tigecycline activity tested against a worldwide collection of *Acinetobacter* spp. (2005—2009). *Diagn Microbiol Infect Dis* 2010;**68**:307—11.
5. Gordon NC, Wareham DW. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents* 2010;**35**:219—26.
6. Esterly J, Richardson CL, Eltoukhy NS, Qi C, Scheetz MH. Genetic mechanisms of antimicrobial resistance of *Acinetobacter baumannii*. *Ann Pharmacother* 2011;**45**:218—28.
7. Hooper DC. Efflux pumps and nosocomial antibiotic resistance: a primer for hospital epidemiologists. *Clin Infect Dis* 2005;**40**:1811—7.
8. Coyne S, Courvalin P, Perichon B. Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob Agents Chemother* 2011;**55**:947—53.
9. Vila J, Marti S, Sanchez-Cespedes J. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2007;**59**:1210—5.
10. Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* 2000;**37**:219—25.
11. Poole K. Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* 2007;**39**:162—76.
12. Magnet S, Courvalin P, Lambert T. Resistance—nodulation—cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother* 2001;**45**:3375—80.
13. Ruzin A, Keeney D, Bradford PA. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus—Acinetobacter baumannii* complex. *J Antimicrob Chemother* 2007;**59**:1001—4.
14. Damier-Piolle L, Magnet S, Bremont S, Lambert T, Courvalin P. AdeIJK, a resistance—nodulation—cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2008;**52**:557—62.
15. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Perichon B. Overexpression of resistance—nodulation—cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2010;**54**:4389—93.
16. Ribera A, Roca I, Ruiz J, Gibert I, Vila J. Partial characterization of a transposon containing the tet(A) determinant in a clinical isolate of *Acinetobacter baumannii*. *J Antimicrob Chemother* 2003;**52**:477—80.
17. Roca I, Marti S, Espinal P, Martinez P, Gibert I, Vila J. CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2009;**53**:4013—4.
18. Rajamohan G, Srinivasan VB, Gebreyes WA. Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2010;**65**:1919—25.
19. Srinivasan VB, Rajamohan G, Gebreyes WA. Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2009;**53**:5312—6.
20. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H^+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother* 2005;**49**:4362—4.
21. Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, et al. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* 2006;**2**:e7.
22. Tanabe M, Szakonyi G, Brown KA, Henderson PJ, Nield J, Byrne B. The multidrug resistance efflux complex, EmrAB from *Escherichia coli* forms a dimer in vitro. *Biochem Biophys Res Commun* 2009;**380**:338—42.
23. Kobayashi N, Nishino K, Yamaguchi A. Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. *J Bacteriol* 2001;**183**:5639—44.
24. Chen TL, Siu LK, Wu RC, Shaio MF, Huang LY, Fung CP, et al. Comparison of one-tube multiplex PCR, automated ribotyping and intergenic spacer (ITS) sequencing for rapid identification of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007;**13**:801—6.
25. Lin MF, Lin YY, Yeh HW, Lan CY. Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii* adeAB genes and its correlation with tigecycline susceptibility. *BMC Microbiol* 2014;**14**:119.
26. Kuo HY, Chang KC, Kuo JW, Yueh HW, Liou ML. Imipenem: a potent inducer of multidrug resistance in *Acinetobacter baumannii*. *Int J Antimicrob Agents* 2012;**39**:33—8.
27. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, et al. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2006;**50**:2946—50.
28. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*. CLSI document M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.
29. Sambrook J, Russell W. *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
30. Ruzin A, Immermann FW, Bradford PA. RT-PCR and statistical analyses of *adeABC* expression in clinical isolates of *Acinetobacter calcoaceticus—Acinetobacter baumannii* complex. *Microb Drug Resist* 2010;**16**:87—9.
31. Pannek S, Higgins PG, Steinke P, Jonas D, Akova M, Bohnert JA, et al. Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-beta-naphthylamide. *J Antimicrob Chemother* 2006;**57**:970—4.
32. Deng M, Zhu MH, Li JJ, Bi S, Sheng ZK, Hu FS, et al. Molecular epidemiology and mechanisms of tigecycline resistance in clinical isolates of *Acinetobacter baumannii* from a Chinese university hospital. *Antimicrob Agents Chemother* 2014;**58**:297—303.
33. Piddock LJ. Multidrug-resistance efflux pumps — not just for resistance. *Nat Rev Microbiol* 2006;**4**:629—36.
34. Kuo SC, Chang SC, Wang HY, Lai JF, Chen PC, Shiau YR, et al. Emergence of extensively drug-resistant *Acinetobacter baumannii* complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program. *BMC Infect Dis* 2012;**12**:200.