Journal of Microbiology (2015) Vol. 53, No. 1, pp. 53–59
DOI 10.1007/s12275-015-4498-5

Effect of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on killing *Acinetobacter baumannii* by colistin

Young Kyoung Park and Kwan Soo Ko*

Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Republic of Korea

(Received Sep 3, 2014 / Revised Nov 6, 2014 / Accepted Nov 12, 2014)

We investigated the effect of cyanide 3-chlorophenylhydrazone (CCCP) and other efflux pump inhibitors (EPIs) on the colistin susceptibility in *Acinetobacter baumannii*. While minimum inhibitory concentrations (MICs) of colistin in all colistin-resistant strains decreased significantly with 25 μM of CCCP and 2,4-dinitrophenol (DNP), phenyl-arginine-β-naphthylamide (PAβN), and reserpine did not decrease the colistin MICs. However, CCCP and DNP as well as PAβN and reserpine did not have a significant effect on the MICs of the other agents. Efflux pump gene expressions in colistin-resistant strains were not increased compared with those in colistin-susceptible strains. When only 5X MIC of colistin (5 mg/L) was provided to a colistin-susceptible *A. baumannii* strain, the bacterial cell number was reduced by 9 h after exposure to colistin, but regrowth was observed. When CCCP was added to colistin, bacterial cells were completely killed after 24 to 48 h of incubation, which was not due to the toxicity of CCCP itself. Colistin resistance in *A. baumannii* may not be due to efflux pumps. Our present study suggests that bacterial cells with reduced metabolic activity by CCCP are more susceptible to colistin in *A. baumannii*. It may show the possibility that combined therapy with colistin and other antimicrobial agents could effective against *A. baumannii* infections.

***Keywords*:** time-kill assay, colistin resistance, efflux pump

Introduction

Acinetobacter baumannii, a nonfermenting, Gram-negative cocobacillus, is one of the important opportunistic pathogens that often infect immunocompromised patients, especially in the intensive care unit. This organism is responsible for a variety of nosocomial infections including bacteremia, urinary tract infections, and pneumonia (Fournier and Richet, 2006). The emergence of multidrug-resistant (MDR) or pandrug-resistant (PDR) *A. baumannii* isolates has become a serious clinical concern worldwide (Munoz-Price and Weinstein, 2008). Colistin has been widely used against *Acinetobacter* infections because of the low resistance rate, and it is now considered one of the last resorts against MDR or PDR *Acinetobacter* infections (Nation and Li, 2009).

Gram-negative bacteria including *A. baumannii* develop resistance to colistin and polymyxin B mainly by modifications of the bacterial outer membrane through alteration of the lipopolysaccharide (LPS) moiety (Falagas *et al.*, 2010). Modifications of the outer membrane decrease the negative surface charge and consequently diminish the affinity of LPS for polymyxins (Lee *et al.*, 2004). Such LPS modification related to alterations to lipid A phosphate groups by the addition of sugar moieties is regulated by a two-component system, PmrAB (Falagas *et al.*, 2010). Although amino acid substitutions in the PmrAB two-component system and elevated expression of *pmrA* have been reported in colistin-resistant *A. baumannii* (Adams *et al.*, 2009), the result that the amino acid changes in the PmrAB two-component system are not essential to colistin resistance was also reported (Park *et al.*, 2011). It was also found that loss of LPS production due to mutations in the genes of lipid A biosynthesis, *lpxA*, *lpxC*, and *lpxD*, contributes the resistance to colistin in *A. baumannii* (Moffatt *et al.*, 2010). Resistance to polymyxins may develop by efflux pump system relating to proton motive force in several bacterial species (Bengoechea and Skurnik, 2000; Pamp *et al.*, 2008). Particularly, Pamp *et al.* (2008) showed that a subpopulation of metabolically active cells in *Pseudomonas aeruginosa* is able to survive the colistin treatment, that is, develop tolerance to colistin. Cells with lower metabolic activity by a metabolic uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were unable to adapt to colistin exposure. Thus, they suggested that combined antimicrobial treatment targeting two physiologically distinct subpopulations such as ciprofloxacin or tetracycline and colistin would completely eradicated *P. aeruginosa* biofilm cells (Pamp *et al.*, 2008).

So far, however, effect of metabolic activity of cell on colistin susceptibility has not been investigated in *A. baumannii*. In this study, we investigated the effect of CCCP and other efflux pump inhibitors (EPIs) on the colistin susceptibility in *A. baumannii*. Metabolically inactive cells became susceptible to colistin, but expression of several known efflux pump genes was not elevated.

Materials and Methods

Bacterial isolates

Six clinical *A. baumannii* strains were collected from Samsung Medical Center (SMC) in Korea and used in this study. Three

*For correspondence. E-mail: ksko@skku.edu; Tel.: +82-31-299-6223; Fax: +82-31-299-6229

Table 1. Primers for qRT-PCR in this study

Gene	Primers	Sequences (5′→3′)
Housekeeping genes		
recA	q-recA-F	5′-ACGGTGAGC AAGCACTTGAA-3′
	q-recA-R	5′-CCGAGTCCACAACGATTAAATCA-3′
gyrB	q-gyrB-F	5′-TTCACAAACAACATTCCACAAAAAG-3′
	q-gyrB-R	5′-GCATCATCACCAGTCAC ATTCA-3′
rpoD	q-rpoD-F	5′-ATTAGAAGCTGCATGGGCTCAA-3′
	q-rpoD-R	5′-GCTTCA TTTGCTTCAGGGCT AT-3′
gdhB	q-gdhB-F	5′-CAGGTTCCTGAAATTGTGAGTG-3′
	q-gdh B-R	5′-GGGTTATGTTTAAAGTCAGGATG-3′
rpoB	q-rpoB-F	5′-GTGCTGACTTGACGCG TGAT-3′
	q-rpoB-R	5′ -AGCGTTCAGAAGAGAAGAACAAGTT-3′
Efflux pump genes		
adeA	q-adeA-F	5′-CCACCACCGGCTAAAGTCAGT-3′
	q-adeA-R	5′-GACTTGCGGACGGATTTCC-3′
adeB	q-adeB-F	5′-TGCCAATTGCACGTTTTCC-3′
	q-adeB-R	5′-TCGCGCTCAATTAAGGTTACAA-3′
adeC	q-adeC-F	5′-GTGATTTACGGACTGCTACGCTTA-3′
	q-adeC-R	5′-GCATTTCCCGTTACACCAATG-3′
adeI	q-adeI-F	5′-GCGTGATTTTAAAACGCTTATTTG-3′
	q-adeI-R	5′-TTGTAGGAGTGATGCTTTTGCATT-3′
adeJ	q-adeJ-F	5′-TGCGTATCTGGCTTGATCCA-3′
	q-adeJ-R	5′-CACCTAACTGACCTACGGCAACT-3′
adeK	q-adeK-F	5′-AGTATGGTCTATTTCAGGTCGTAGCAT-3′
	q-adeK-R	5′-CGGTTTTCACGACTGGTTCTG-3′
adeR	q-adeR-F	5′-TGAGTGTTATTCGGGCCATGA-3′
	q-adeR-R	5′-CTTCCCAGCCGTTTAATTCG-3′
adeS	q-adeS-F	5′-ATGCGCCTCGCAAAGC-3′
	q-adeS-R	5′-CATTTCGGCGGAGTGAATTC-3′

strains (07AC-052, 07AC-111, and 07AC-347) were wild-type colistin-resistant and colistin resistance of the other three mutant strains (06AC-06-R1, 06AC-08-R1, and 06AC-17-R1) were induced *in vitro* from three susceptible parent strains (06AC-06, 06AC-08, and 06AC-17) in cation-adjusted Mueller-Hinton broth incorporating increasing concentrations of colistin from 1 mg/L to 16 mg/L (Park *et al.*, 2011). The minimum inhibitory concentrations (MICs) of colistin of all colistin-susceptible strains were 1 mg/L, and those of colistin-resistant strains were 16 mg/L (07AC-111) and >64 mg/L (the others) (Table 2).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). MICs of seven antimicrobial agents including imipenem, colistin, tetracycline, ciprofloxacin, amikacin, cefepime, and tigecycline were determined. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. To examine an effect of efflux pumps in antimicrobial resistance, MICs were measured in the presence of various EPIs. 25 mg/L of phenyl-arginine-β-naphthylamide (PAβN) (Peleg *et al.*, 2007), 10 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), 1 mM of 2,4-dinitrophenol (DNP), and 20 mg/L of reserpine were added into cation-adjusted Mueller-Hinton broth and used for MIC testing.

Quantitative reverse transcription PCR (qRT-PCR)

To examine the change of bacterial cell activity when exposed to CCCP, expressions of four housekeeping genes such as *recA*, *gyrB*, *rpoD*, and *gdhB* were evaluated in colistin-susceptible (06AC-06) and colistin-resistant (07AC-111) isolates. After exposure of bacterial cells to colistin (5 mg/L) or colistin (5 mg/L) + CCCP (10 μM) for 3 h by qRT-PCR. In addition, expression level of *pmrAB* two-component regulatory system was determined at the same time. The expressions of well-known efflux pump genes such as *adeABC*, *adeIJK*, and *adeRS* were compared between colistin-resistant and -susceptible strains. Total RNA was isolated from the mid-

Table 2. Colistin MICs of wild-type and derived colistin-resistant strains when treated with EPIs

Strain	Colistin MIC (mg/L)[a]								
	Only colistin	Colistin + CCCP (10 μM)		Colistin + DNP (1 mM)		Colistin + PAβN (25 mg/L)		Colistin + Reserpine (20 mg/L)	
		MIC	Fold change	MIC	Fold change	MIC	Fold change	MIC	Fold change
Colistin-susceptible									
06AC-06	1								
06AC-08	1								
06AC-17	1								
Induced colistin-resistant									
06AC-06-R1	>64	1	[>64]	2	[>32]	>64	-	>64	-
06AC-08-R1	>64	0.5	[>128]	1	[>64]	>64	-	>64	-
06AC-17-R1	>64	0.125	[>512]	4	[>16]	>64	-	>64	-
Wild-type colistin-resistant									
07AC-052	>64	0.5	[>128]	2	[>32]	>64	-	>64	-
07AC-111	16	0.25	[64]	2	[8]	16	-	16	-
07AC-347	>64	0.5	[>128]	4	[>16]	>64	-	>64	-

[a] CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; PAβN, phenyl-arginine-β-naphthylamide
-, no fold change

Table 3. MICs of wild-type and induced colistin-resistant strains when treated with EPIs

Antimicrobial agent	Isolate	MIC (mg/L)[a]				
		Antibiotic only	CCCP (10 μM)	DNP (1 mM)	PAβN (25 mg/L)	Reserpine (20 mg/L)
Colistin	06AC-06-R1	>64	1	2	>64	>64
	06AC-08-R1	>64	0.5	1	>64	>64
	06AC-17-R1	>64	0.125	1	>64	>64
	07AC-052	>64	0.5	2	>64	>64
	07AC-111	16	0.25	2	16	16
	07AC-347	>64	0.5	4	>64	>64
Imipenem	06AC-06-R1	1	1	4	4	2
	06AC-08-R1	2	2	4	2	2
	06AC-17-R1	16	16	16	16	16
	07AC-052	>64	>64	>64	>64	>64
	07AC-111	0.125	0.5	0.125	0.125	0.125
	07AC-347	>64	>64	>64	>64	>64
Tigecycline	06AC-06-R1	4	2	2	4	4
	06AC-08-R1	4	2	2	4	4
	06AC-17-R1	4	4	2	4	4
	07AC-052	4	4	2	4	8
	07AC-111	0.125	0.125	0.125	0.06	0.5
	07AC-347	4	4	2	4	4
Ciprofloxacin	06AC-06-R1	>64	>64	>64	>64	>64
	06AC-08-R1	>64	>64	>64	>64	>64
	06AC-17-R1	>64	>64	>64	>64	>64
	07AC-052	>64	>64	>64	>64	>64
	07AC-111	0.25	0.25	0.25	0.25	0.25
	07AC-347	>64	>64	>64	>64	>64
Amikacin	06AC-06-R1	>64	>64	>64	>64	>64
	06AC-08-R1	>64	>64	>64	>64	>64
	06AC-17-R1	>64	>64	>64	>64	>64
	07AC-052	>64	>64	>64	>64	>64
	07AC-111	2	2	4	4	8
	07AC-347	>64	>64	>64	>64	>64
Cefepime	06AC-06-R1	>64	>64	>64	>64	>64
	06AC-08-R1	>64	>64	64	>64	>64
	06AC-17-R1	>64	>64	>64	>64	>64
	07AC-052	>64	>64	>64	>64	16
	07AC-111	1	1	1	1	1
	07AC-347	>64	>64	>64	>64	>64
Tetracyclin	06AC-06-R1	16	16	16	32	32
	06AC-08-R1	16	16	16	32	32
	06AC-17-R1	16	16	16	32	16
	07AC-052	16	8	8	16	16
	07AC-111	0.5	0.5	0.5	0.5	0.5
	07AC-347	16	16	8	16	16

[a] CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; PAβN; phenyl-arginine-β-naphthylamide.

log phase bacterial culture (OD_{600}=0.5) by using an RNeasy mini kit (Qiagen). The RNA sample was treated further with RNase-free DNase (Invitrogen). Transcripts were quantified by real-time PCR using SYBR Premix Ex Tag (TaKaRa) on Thermal Cycler Dice Real Time System (TaKaRa). Transcription levels of housekeeping genes and the efflux pump genes were normalized to *rpoB*-encoded gene expression levels. Primer sets used in this study are listed in Table 1. All tests were done in duplicate. Student's *t*-test was used to determine the significant differences in gene expression. All data were analyzed by using the SPSS 12 for Windows. Differences were considered statistically significant at a *P* value of ≤0.05.

In vitro time-kill assay with CCCP

In vitro time-kill assay was performed for three colistin-susceptible *A. baumannii* strains. For *in vitro* time-kill assay, the bacterial cells of 06AC-06 were grown for overnight in LB broth medium and cells of stationary phase were re-inoculated 1:100 in fresh LB containing 5 mg/L of colistin (5X MIC) with or without CCCP (10 μM, 25 μM, and 50 μM).

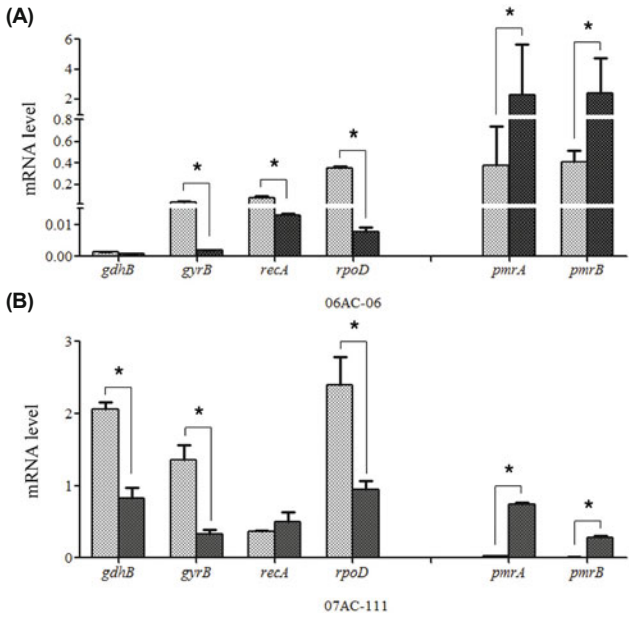

Fig. 1. Comparison of expression of housekeeping genes and *pmrAB* in (A) a colistin-susceptible strain 06AC-06 and in (B) a colistin-resistant *A. baumannii* strain 07AC-111. Gray, treatment of only colistin (5 mg/L); black, treatment of colistin (5 mg/L) and CCCP (10 μM). *, $P<0.05$.

The cultures were incubated for 48 h. During incubation 3, 6, 9, 24, and 48 h samples were obtained and spread on LB agar plate. Plates were grown for 24 h before colony counting. To explore the effect of CCCP itself on bacterial cell death, an *in vitro* time-kill assay was performed using media containing only 50 μM of CCCP (no colistin). In addition, bacterial cell growth after pre-exposure to 10 μM of CCCP was investigated to assess the time interval required until the CCCP functions. After a strain 06AC-06 was grown overnight with CCCP, an *in vitro* time-kill experiment was performed. For the other two colistin-susceptible strains (06AC-08 and 06AC-17), *in vitro* time-kill analyses were performed using 5 mg/L of colistin and 10 μM of CCCP based on the results of the strain 06AC-06. Also, time-killing curves were determined when CCCP (5 μM and 10 μM) was added after 6 h incubation with colistin in all the three isolates.

Results

Colistin resistance and efflux pump activity

Colistin-resistant derivatives showed very high colistin MICs (>64 mg/L) compared with their parent strains (1 mg/L) (Table 2). The changes of colistin MICs in colistin-resistant *A. baumannii* strains by EPIs are shown in Table 2. Colistin MICs of all colistin-resistant strains decreased significantly with 10 μM of CCCP from 64-fold in 07AC-111 to >512-fold in 06AC-17-R1. DNP also decreased significantly the colistin MICs in six colistin-resistant strains (MICs, 1 to 4 mg/L), although the reduced range was not comparable with CCCP. However, PAβN and reserpine did not decrease the colistin MICs of colistin-resistant strains.

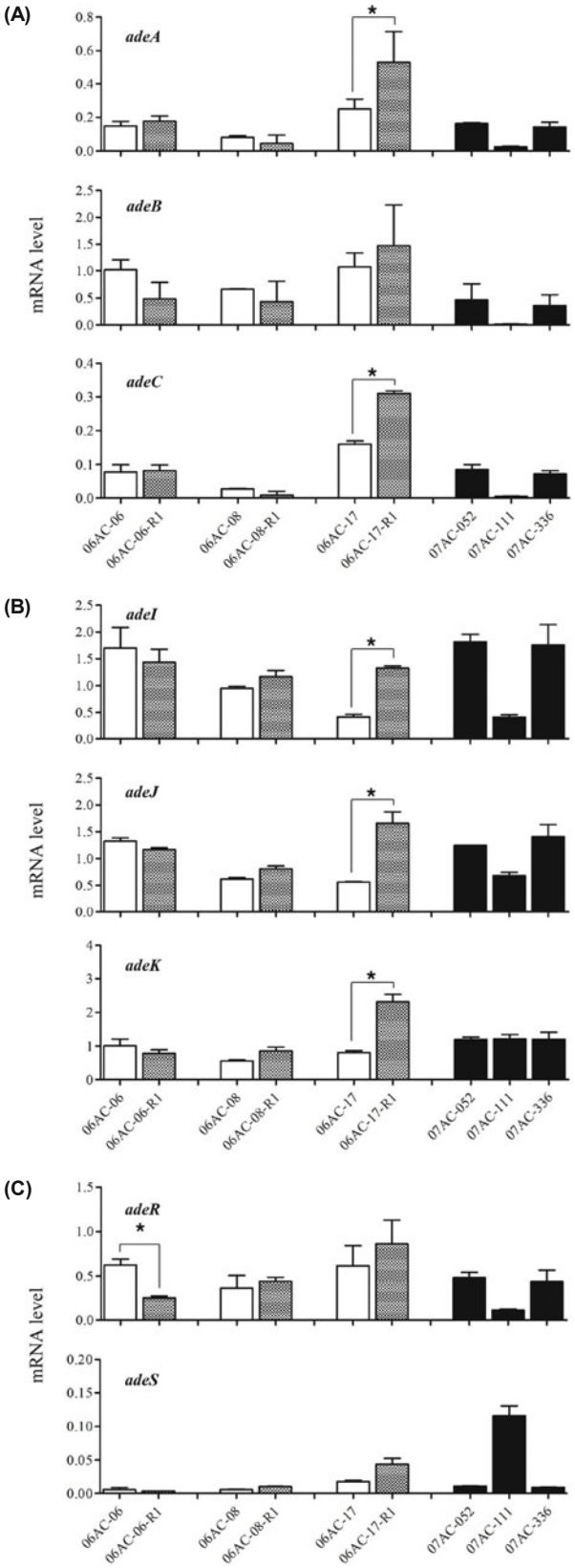

Fig. 2. Expression of efflux pump genes. (A) *adeABC*, (B) *adeIJK*, (C) *adeRS*. White, colistin-susceptible parent strain; gray, colistin-resistant derivative from susceptible parent strain; black, wild-type colistin-resistant strain. *, $P<0.05$.

The effect of EPIs on the MICs of the other six antimicrobial agents was also examined in all colistin-resistant strains (Table 3). CCCP and DNP, which had distinct effects on colistin MIC, did not significantly affect the MICs of the other agents. MICs of few antimicrobial agents were slightly changed with EPIs in some cases, but those were not that significant.

Expression of housekeeping, efflux pump genes, and *pmrAB* two-component system

We compared cell metabolic activity of a colistin-susceptible strain 06AC-06 (Fig. 1A) and colistin-resistant strain 07AC-111 (Fig. 1B) by evaluating the expression of housekeeping genes. qRT-PCR results showed significant decreased expression of *gyrB* and *rpoD* when the both strains were grown with the combination of colistin and CCCP. The expression of *gdhB* and *recA* was respectively reduced in 07AC-111 and 06AC-06. The reduction of three housekeeping genes may indicate overall reduction of metabolic and physiological activity by CCCP treatment. In contrast, the expression levels of *pmrAB* two-component system were highly increased in cells grown with colistin and CCCP together (Fig. 1).

Efflux pump gene expressions in colistin-resistant strains were not increased compared with those in colistin-susceptible strains (Fig. 2). Among three *in vitro* derived colistin-resistant strains, only 06AC-17-R1 showed increased expression of *adeABC*, compared with its colistin-susceptible parent strain (Fig. 2A). Wild-type colistin-resistant strains also showed no higher expression of *adeABC* than colistin-susceptible strains. In *adeIJK*, only 06AC-17-R1 showed significantly higher expression than its colistin-susceptible parent strain as in *adeABC* (Fig. 2B). Wild-type colistin-resistant strains showed greater expression of *adeJ* than two susceptible strains (06AC-08 and 06AC-17), but not than another susceptible strain, 06AC-06. In addition, consistent elevated expression in colistin-resistant strains was also not observed in *adeRS* (Fig. 2C). Although 07AC-17-R1 showed higher gene expression of *adeRS* compared its colistin-susceptible parent strain, the other induced colistin-resistant strains did not. A wild-type resistant strain 07AC-111 showed generally lower expression levels of efflux pump genes except *adeS* than the other wild-type resistant strains, which may be associated with relatively low MIC of colistin (16 mg/L).

In vitro time-kill analysis

Figure 3 shows the results of *in vitro* time-kill analyses using colistin and CCCP. When only 5X MIC of colistin (5 mg/L) was to a colistin-susceptible *A. baumannii* strain 06AC-06, the bacterial cell numbers were reduced by 9 h after exposure to colistin (Fig. 3A). However, regrowth was observed after

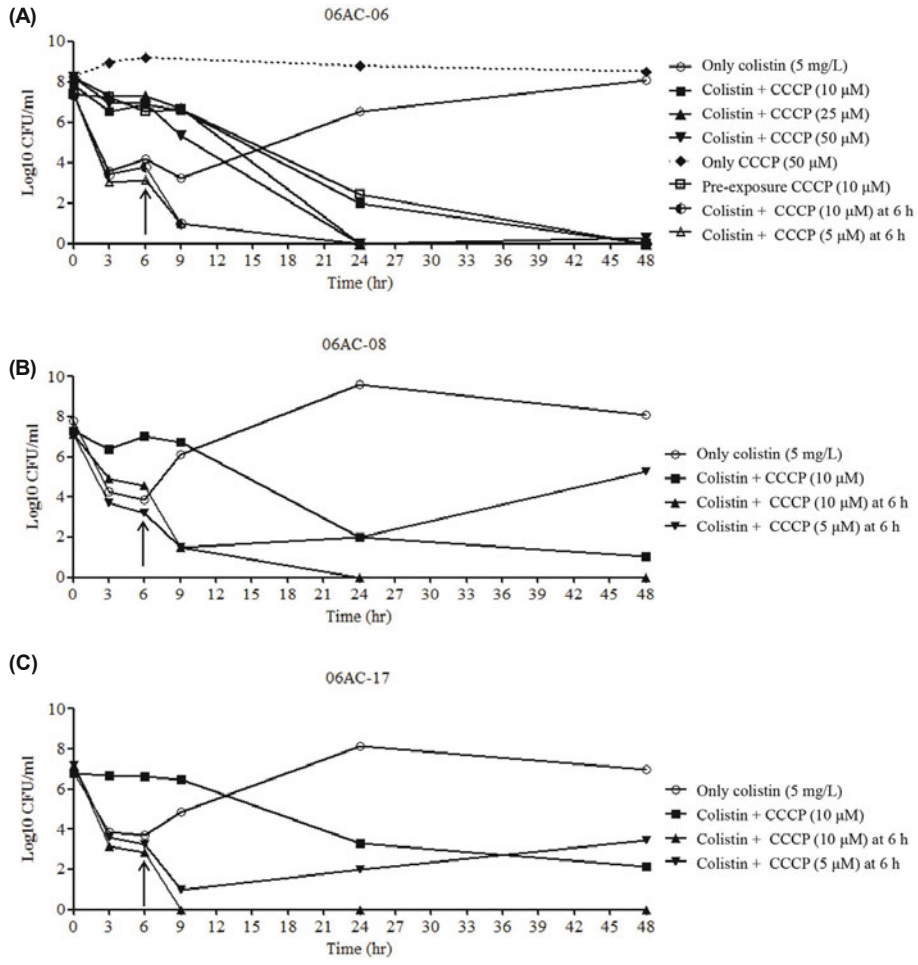

Fig. 3. *In vitro* time-kill curves in colistin-susceptible *A. baumannii* strains. In all experiments except that using only CCCP, 5 mg/L of colistin was treated. (A) 06AC-06, (B) 06AC-08, (C) 06AC-17. Arrow, indicating the addition of CCCP.

24 h of exposure to colistin. Thus, a single regimen of colistin could not inhibit the growth of *A. baumannii* completely. When CCCP was added to colistin, bacterial cells were completely killed after 24 to 48 h of incubation. However, the killing effect of additional CCCP was observed only a little until 9 h of incubation, which was comparable with the result of treatment of only colistin.

When only CCCP (50 μM) was treated, the bacterial cell growth was not inhibited, which indicated that inhibition of bacterial cell growth by addition of CCCP to colistin was not due to the toxicity CCCP itself. In addition, pre-exposure to CCCP (10 μM) did not affect the *in vitro* time-kill curve. The other two colistin-susceptible strains showed the same pattern with 06AC-06; regrowth in only colistin and early retardation of bacterial killing and additional killing effect by addition of CCCP (Fig. 3B and C).

We also determined time-killing curves when bacterial cells were exposed to CCCP (10 μM) after 6 h colistin treatment. Cells were killed more clearly compared with cells treated to colistin and CCCP initially. However, regrowth was observed when we repeated same experiments using less CCCP (5 μM).

Discussion

Polymyxins including colistin targets LPS of cell membrane. Having a positive charge, it displaces Mg^{2+} or Ca^{2+} and binds on lipid A component, resulting in the destabilization and disruption of the bacterial membrane (Falagas *et al.*, 2010). Thus, it can be potent to cells with low metabolic activity. Pamp *et al.* (2008) suggested that colistin preferably killed biofilm cells with low metabolic and physiological activity and that biofilm cells lacking metabolic and physiological activity by CCCP were sensitive to colistin. Our present study also suggests that bacterial cells with reduced metabolic activity by CCCP are more susceptible to colistin in *A. baumannii*.

Disruption of proton motive force by CCCP does not only inhibit metabolic activity, but also inhibits H^+- and ATP-driven efflux pumps. Pamp *et al.* (2008) showed that CCCP treatment interfered with the function of efflux pumps and rendered *P. aeruginosa* susceptible to colistin and that *mexAB-oprM* mutants exhibited increased susceptibility to colistin. However, our results that CCCP and DNP reduced the colistin MICs but PAβN and reserpine did not reduce them may indicate that efflux pumps themselves do not contribute directly to the resistance to colistin in *A. baumannii*. Although CCCP and DNP are inhibitors of efflux pumps, they differ as uncouplers of oxidative phosphorylation (Nakajima, 1999), from PAβN and reserpine, competitive EPIs. In qRT-PCR experiments on proton motive force-dependent resistance-modulation-cell division (RND) type efflux pump genes, elevated expressions could not be identified in all colistin-resistant *A. baumannii* strains. Thus, the effect of CCCP or DNP reducing the colistin MICs may not be related with inhibition of efflux pumps, and colistin resistance in *A. baumannii* may not be due to efflux pumps.

Retardation of bacterial killing at an early stage in *in vitro* time-kill experiments was unexpected, which was consistent in three *A. baumannii* strains. Pre-exposure to CCCP was not required to remove the delayed start of killing effect. CCCP disrupts the electrochemical gradient across the bacterial membrane. Such depolarized membrane by CCCP may protect cells from colistin until removal of the uncoupler (Alteri *et al.*, 2011). Another possibility is that the increase of *pmrAB* gene expressions when treated to colistin and CCCP together may influence their survival. Higher expression of *pmrAB* might occur some changes in bacterial cell membrane and inhibit the interaction of membrane and colistin.

The clinical implications of our results are obvious. As in this study, regrowth after very rapid initial killing by polymyxins is frequently observed, and the surviving bacterial cells often have significantly higher MICs (Landman *et al.*, 2008). The re-growth observed in our results of time-kill assay was thought to be due to resistant-subpopulation survived against colistin treatment. It has repeatedly seen that monotherapy with colistin may be problematic for the treatment of *A. baumannii* infections (Owen *et al.*, 2007). As indicated by many authors, the combined treatment with colistin and another antimicrobial agent would also be very effective, eradicating both active and dormant bacterial cell populations in *A. baumannii* (Landman *et al.*, 2008).

In this study, we found that colistin is more active against bacterial cells with reduced metabolic activity by CCCP in *A. baumannii*. Our findings may show the possibility that combined therapy with colistin and other antimicrobial agents could effective against *A. baumannii* infections. Although all known mechanisms of colstin resistance in *A. baumannii* were related with membrane modifications, our results could provide one of new concept for study on colistin resistance mechanisms.

Acknowledgements

All *A. baumannii* strains used in this study were obtained from the Asian Bacterial Bank (ABB) of the Asia Pacific Foundation for Infectious Disease (APFID) (Seoul, Republic of Korea). Park Y.K. was supported partly by Basic Science Research Program through the NRF funded by the Ministry of Education (2013R1A1A2060064).

References

Adams, M.D., Nickel, G.C., Bajaksouzian, S., Lavender, H., Murthy, A.R., Jacobs, M.R., and Bonomo, R.A. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob. Agents Chemother.* **53**, 3628–3634.

Alteri, C.J., Lindner, J.R., Reiss, D.J., Smith, S.N., and Mobley, H.L.T. 2011. The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli*. *Mol. Microbiol.* **82**, 145–163.

Bengoechea, J.A. and Skurnik, M. 2000. Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in Yersinia. *Mol. Microbiol.* **37**, 67–80.

Clinical and Laboratory Standards Institute (CLSI). 2013. Performance Standards for Antimicrobial Susceptibility Testing, Twenty-First Informational Supplement. CLSI Document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Falagas, M.E., Fafailidis, P.I., and Matthaiou, D.K. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist. Updates* **13**, 132–138.

Fournier, P.E. and Richet, H. 2006. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin. Infect. Dis.* **42**, 692–699.

Landman, D., Georgescu, C., Martin, D.A., and Quale, J. 2008. Polymyxins revisited. *Clin. Microbiol. Rev*. **21**, 449–465.

Lee, H., Hsu, F.F., Turk, J., and Groisman, E.A. 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J. Bacteriol.* **186**, 4124–4133.

Moffatt, J.H., Harper, M., Harrison, P., Hale, J.D., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St Michael, F., Cox, A.D., *et al.* 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide. *Antimicrob. Agents Chemother*. **54**, 4971–4977.

Munoz-Price, L.S. and Weinstein, R.A. 2008. *Acinetobacter* infection. *N. Engl. J. Med.* **358**, 1271–1281.

Nakajima, Y. 1999. Mechanisms of bacterial resistance to macrolide antibiotics. *J. Infect. Chemother.* **5**, 61–74.

Nation, R.L. and Li, J. 2009. Colistin in the 21st century. *Curr. Opin. Infect. Dis.* **22**, 535–543.

Owen, R.J., Li, J., Nation, R.L., and Spelman, D. 2007. *In vitro* pharmacodynamics of colistin against *Acinetobacter baumannii* clinical isolates. *J. Antimicrob. Chemother.* **59**, 473–477.

Pamp, S.J., Gjermansen, M., Johansen, H.K., and Tolker-Nielsen, T. 2008. Tolerance to the antimicrobial peptide colisitn in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol. Microbiol.* **68**, 223–240.

Park, Y.K., Choi, J.Y., Shin, D., and Ko, K.S. 2011. Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents* **37**, 525–530.

Peleg, A.Y., Adams, J., and Paterson, D.L. 2007. Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. **51**, 2065–2069.