Journal of Microbiology (2015) Vol. 53, No. 4, pp. 256–261
DOI 10.1007/s12275-015-5037-0

Functional analysis of *Vibrio vulnificus* RND efflux pumps homologous to *Vibrio cholerae* VexAB and VexCD, and to *Escherichia coli* AcrAB

Seunghwa Lee[1†], Ji-Hyun Yeom[1†], Sojin Seo[1], Minho Lee[1], Sarang Kim[2], Jeehyeon Bae[2*], Kangseok Lee[1*], and Jihwan Hwang[3*]

[1]*Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea*
[2]*College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea*
[3]*Department of Microbiology, Pusan National University, Busan 609-735, Republic of Korea*

(Received Jan 21, 2015 / Revised Jan 28, 2015 / Accepted Jan 29, 2015)

Resistance-nodulation-division (RND) efflux pumps are associated with multidrug resistance in many gram-negative pathogens. The genome of *Vibrio vulnificus* encodes 11 putative RND pumps homologous to those of *Vibrio cholerae* and *Escherichia coli*. In this study, we analyzed three putative RND efflux pumps, showing homology to *V. cholerae* VexAB and VexCD and to *E. coli* AcrAB, for their functional roles in multidrug resistance of *V. vulnificus*. Deletion of the *vexAB* homolog resulted in increased susceptibility of *V. vulnificus* to bile acid, acriflavine, ethidium bromide, and erythromycin, whereas deletion of *acrAB* homologs rendered *V. vulnificus* more susceptible to acriflavine only. Deletion of *vexCD* had no effect on susceptibility of *V. vulnificus* to these chemicals. Upon exposure to these antibacterial chemicals, expression of *tolCV1* and *tolCV2*, which are putative outer membrane factors of RND efflux pumps, was induced, whereas expression levels of *vexAB*, *vexCD*, and *acrAB* homologs were not significantly changed. Our results show that the *V. vulnificus* homologs of VexAB largely contributed to *in vitro* antimicrobial resistance with a broad substrate specificity that was partially redundant with the AcrAB pump homologs.

Keywords: AcrAB, multidrug resistance, resistance-nodulation-division, VexAB, VexCD

Introduction

Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Vibrio cholerae* use tripartite efflux pumps to extrude antibacterial drugs and other toxic compounds from the inner cytoplasm or periplasmic space to the outer environment. Well-studied examples of efflux pumps are the *E. coli* MacAB-TolC and AcrAB-TolC pumps, and the *P. aeruginosa* MexAB-OprM pumps (Zgurskaya and Nikaido, 1999; Koronakis *et al*., 2004).

Tripartite efflux pumps are typically comprised of an outer membrane factor (OMF), a periplasmic membrane fusion protein (MFP), and an inner membrane transporter (IMT). The IMT belongs to one of three structurally dissimilar protein super-families, including the major facilitator family, resistance-nodulation-cell division (RND) family, and the ATP binding cassette (ABC) family (Misra and Bavro, 2009). *E. coli* shares a single OMF encoded from a *tolC* allele, whereas many other bacteria encode cognate TolC homologs for their tripartite efflux systems (Li *et al*., 1995; Poole *et al*., 1996; Kohler *et al*., 1997; Bina *et al*., 2000; Song *et al*., 2014). The OMF connects to the IMT through MFP in the periplasmic space, providing a continuous conduit to the extracellular environment. Several models for the assembly of the tripartite pump have focused on the direct interaction between the IMT AcrB and the OMF TolC, which was detected only when cross-linking was induced (Tamura *et al*., 2005; Bavro *et al*., 2008; Symmons *et al*., 2009). However, the mutants that were used for *in vivo* cross-linking appeared to be nonfunctional (Kim *et al*., 2010; Xu *et al*., 2011b), and therefore, these studies need to be reevaluated. In contrast, a recent assembly model for tripartite efflux pumps proposes a direct interaction between the α-barrel of MFPs with the α-barrel of OMFs in a tip-to-tip manner, like intermeshing cogwheels (Kim *et al*., 2010; Xu *et al*., 2011a, 2011b; Lee *et al*., 2012, 2013, 2014; Song *et al*., 2014). A conserved structural motif at the α-barrel of MFPs called the RLS motif has been shown to be important for this interaction. In addition, direct interaction between AcrA and TolC has recently been confirmed by crystal structures of the AcrAB-TolC pump (Du *et al*., 2014).

RND-type efflux pumps are important for survival of pathogenic bacteria, particularly during infections. Six RND type efflux pumps have been identified in the human pathogenic bacterium *V. cholerae* (Bina *et al*., 2006; Rahman *et al*., 2007), whereas the marine pathogenic bacterium *V. vulnificus* encodes 11 putative RND pumps that are homologous to those of *V. cholerae* and *E. coli* (Park *et al*., 2011; Kawano *et al*., 2014). However, little is known about the functional role of these putative *V. vulnificus* RND pumps. In this study, we analyzed the functional role of *V. vulnificus* RND efflux pumps that were composed of MFPs with an RLS-like motif, which are homologous to *V. cholerae* VexAB and VexCD and to *E. coli* AcrAB.

[†]These authors contributed equally to this work.
*For correspondence. (K. Lee) E-mail: kangseok@cau.ac.kr; Tel.: +82-2-822-5241; Fax: +82-2-825-5026 / (J. Bae) E-mail: jeehyeon@cau.ac.kr; Tel.: +82-2-820-5604; Fax: +82-2-820-5091 / (J. Hwang) E-mail: hwangjh@pusan.ac.kr; Tel.: +82-51-510-2194; Fax: +82-51-514-1778

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Deletion of *vexAB*, *vexCD*, and *acrAB1* genes in *V. vulnificus*

Regions of the *vexAB* untranslated region (UTR) (upstream 750 bp, downstream 750 bp), *vexCD* UTR (upstream 750 bp, downstream 750 bp), and *acrAB1* UTR (upstream 750 bp, downstream 750 bp) were amplified by overlap extension polymerase chain reaction (PCR) from *V. vulnificus* MO6-24/O genomic DNA using the following 12 primers: *vexAB*-5′UTR-F (5′-TTCTGCAGCTCATTGACAAGATTTTTAC-3′), *vexAB*-5′UTR-R (5′-CGTTTATATTGCATCGCAATC TATTAAGAACTCCAATT-3′), *vexAB*-3′UTR-F (5′-AATTG GAGTTCTTAATAGATTGCGATGCAATATAAACG-3′), *vexAB*-3′UTR-R (5′-AAACTAGTGACACTCATTGCCGT AAATG-3′), *vexCD*-5′UTR-F (5′-TTCTGCAGCTTACGA TTTACGCTTGCCCAT-3′), *vexCD*-5′UTR-R (5′-CAGAC CGAACATGATGGTGTGCATCCCACCACCAGCAA-3′), *vexCD*-3′UTR-F (5′-TTGCTGGTGGTGGGATGCACACC ATCATGTTCGGTCTG-3′), *vexCD*-3′UTR-R (5′-AAACT AGTTTCTTGCAGACGCTGCAATCCT-3′), *acrAB1*-5′UTR-F (5′-TTCTGCAGGTTTGAATAATCTTT GCAGCA-3′), *acrAB1*-5′UTR-R (5′-TGGGATCAAGAAGGTACCGGCC AACAGAGCCGAAGCCA-3′), *acrAB1*-3′UTR-F (5′-TGG CTTCGGCTCTGTTGGCCGGTACCTTCTTGATCCCA-3′), and *acrAB1*-3′UTR-R (5′-AAACTAGTTGGCGGCGCGG TCGAGATCAT-3′). PCR products were digested with *Pst*I and *Spe*I and ligated into the same sites of vector pSW7848 to produce pSW7848-*vexAB*, -*vexCD*, and -*acrAB1* UTRs. These constructs were used to transform β3914 cells, and transformants were selected from LB plates containing 300 μM diaminopimelic acid and 20 μg/ml chloramphenicol. Overnight cultures of donor and recipient cells were diluted 1:100 in culture medium without antibiotics and grown at 30°C in LB medium supplemented with an additional 2% NaCl (LBS) to an OD_{600} of 0.6. Experiments were performed using a mating procedure with a donor to recipient ratio of 1:10. Conjugation was performed overnight on LB agar medium supplemented with diaminopimelic acid and 2% NaCl at 30°C. Counterselection of *dapA* donors was performed by plating on a medium lacking diaminopimelic acid but supplemented with 5 μg/ml chloramphenicol. Antibiotic-resistant colonies were isolated and grown in LB medium containing 2% NaCl to late logarithmic phase and spread on plates containing 0.2% arabinose. Mutants were screened by PCR using primers -5′UTR-F and -3′UTR-R of each targeted operon.

Measurement of minimum inhibitory concentration (MIC)

The procedure for MIC measurement has been previously described (Lee *et al.*, 2012, 2013). Briefly, overnight cultures grown in LBS medium were diluted 1:100 in the same medium. At an optical density at 600 nm (OD_{600}) of 0.6, approximately 10^4 cells were added to the same medium containing acriflavine, bile acid, erythromycin, ethidium bromide (EtBr), novobiocine, and sodium dodecyl sulfate (SDS) in increasing concentrations. The cultures were grown for an additional 16 to 18 h, and the lowest concentrations of these chemicals that completely inhibited growth were designated as the MICs.

RNA preparation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The procedure for RT-PCR has been previously described (Lim *et al.*, 2014). Overnight cultures grown in LBS medium were diluted 1:100 in the same medium. At an OD_{600} of 0.1, each chemical was added at 10 and 20 μg/ml for bile acid, 1 and 2 μg/ml for acriflavine, and 0.5 and 1 μg/ml for erythromycin to the cultures, and they were incubated for 2 more hours. Total RNA was isolated using RNeasy Miniprep Kit (Qiagen). One μg of total RNA from each sample was used for cDNA synthesis using PrimeScript 1st strand cDNA Synthesis Kit for RT-PCR (TaKaRa). The primers used for RT-PCR were 5′-AGCAAAACGATCCACAGTTATTGA-3′ and 5′-ACGTTCAGGTTTTTATGCTCTTGA-3′ for *tolCV1*, 5′-AAGCTAAACAAAACGATCCGAATT-3′ and 5′-TCA GAGTGTCGGTGCGAATTTGAT-3′ for *tolCV2*, 5′-TGA AATGACCAATACCGTCGTAAC-3′ and 5′-ACTGCCGT TGTACAGGACAAATTC-3′ for *vexAB*, 5′-GATTTGAAG ACCCTGAATTTGTCA-3′ and 5′-CAACAGTAATCAGG

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	References
E. coli		
π3813	B462 Δ*thyA*::(*erm-pir*-116), Em^r	Le Roux *et al.* (2007)
β3914	F-RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*) *gyrA462 zei-298*::Tn*10*, Km^r, Em^r, Tc^r	Le Roux *et al.* (2007)
V. vulnificus		
MO6-24/O	Clinical isolate	Wright *et al.* (1985)
Δ*vexAB*	MO6-24/O, *vexAB* deleted	This study
Δ*vexCD*	MO6-24/O, *vexCD* deleted	This study
Δ*acrAB1*	MO6-24/O, *acrAB1* deleted	This study
Plasmids		
pSW7848	A *ccdB* containing derivative of pSW4426T ; R6K *ori*V, RP4 *oriT*, *ccdB-araC*; Pir dependent replication, Cm^r	Val *et al.* (2008)
pSW7848-*vexAB* UTR	pSW7848 with 1.5 kb upstream and downstream region of *vexAB*, Cm^r	This study
pSW7848-*vexCD* UTR	pSW7848 with 1.5 kb upstream and downstream region of *vexCD*, Cm^r	This study
pSW7848-*acrAB1* UTR	pSW7848 with 1.5 kb upstream and downstream region of *acrAB1*, Cm^r	This study

Table 2. Putative RND-type efflux systems in *V. vulnificus* MO6-24/O

VV gene	Identity against		Designation	Characteristics
	E. coli AcrA or AcrB	*V. cholerae* pumps		
VVMO6_00057	27%	83%	VexA	MFP / conserved RLS motif
VVMO6_00056	33%	88%	VexB	IMT
VVMO6_03480	25%	52%	VexC	MFP / conserved RLS motif
VVMO6_03481	25%	81%	VexD	IMT
VVMO6_00577	very low	61%	VexE	MFP
VVMO6_00578	24%	84%	VexF	IMT
VVMO6_02100	25%	68%	VexG	MFP
VVMO6_02099	30%	86%	VexH	IMT
VVMO6_01821	25%	72%	VexI	MFP
VVMO6_01820	24%	65%	VexJ	MFP
VVMO6_01819	23%	88%	VexK	IMT
VVMO6_03819	29%	66%	VexL	MFP
VVMO6_03818	26%	82%	VexM	IMT
VVMO6_03994	34%	n.d.	AcrA1	MFP / conserved RLS motif
VVMO6_03993	39%	n.d.	AcrB1	IMT
VVMO6_03693	24%	n.d.	AcrA2	MFP / conserved RLS motif
VVMO6_03694	24%	n.d.	AcrA2	MFP / conserved RLS motif
VVMO6_03695	24%	n.d.	AcrB2	IMT
VVMO6_04219	26%	n.d.	AcrA3	MFP
VVMO6_04220	25%	n.d.	AcrB3	IMT
VVMO6_04320	22%	n.d.	AcrA4	MFP
VVMO6_04319	22%	n.d.	AcrB4	IMT
VVMO6_03130	22%	n.d.	AcrA5	MFP / conserved RLS motif
VVMO6_03129	22%	n.d.	AcrB5	IMT

* n. d.; not determined

TTTTGCTTCT-3′ for *vexCD*, 5′-GAACATGATCTACAT GTCGTCCAA-3′ and 5′-TAAACGCTTGAACCATCTGA ATTG-3′ for *acrAB1*, and 5′-TTGATTGGCCAGAATTGG AGTTTG-3′ and 5′-TGTGGTCGTCGGGATCAAGCTCA T-3′ for *gapNAD* as an endogenous loading control (Kim *et al.*, 2013).

Detection of total protease activity

Total protease activity was observed using a 1.5% skim milk-agar plate, as previously described (Hwang *et al.*, 2011). Briefly, overnight cultures grown in LBS medium were diluted 1:100 in the same medium. At an optical density at 600 nm (OD_{600}) of 0.6, approximately 10^4 cells were spotted on LBS-agar plates containing 1.5% skim milk. The cleared zones around bacterial colonies were monitored after 16 h of incubation at 30°C.

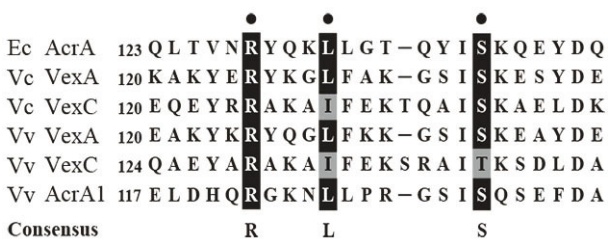

Fig. 1. Sequence comparison of the α-hairpins with the antiparallel coiled-coil RLS motif (Ec, *E. coli*; Vc, *V. cholera*; and Vv, *V. vulnificus*).

Results

Sequence analysis of putative RND pumps in *V. vulnificus*

The genome of *V. vulnificus* contains DNA segments encoding genes for 11 putative RND pumps homologous to those of *V. cholerae* and *E. coli* (Table 2). All of these homologs showed an amino acid sequence similarity to *E. coli* AcrA and AcrB that ranged from ~20% to ~40%. We, therefore, tentatively assigned those showing a higher sequence homology (> ~50%) to *V. cholerae* RND pumps to *V. cholerae* nomenclature, designated by VexA-M. Those having no sequence homology to *V. cholerae* RND pumps were designated as AcrAB1-AcrAB5. Further sequence analysis of these putative RND pumps indicated the existence of putative RLS motifs in MFP proteins homologous to *V. cholerae* VexA and VexC and to *E. coli* AcrA (Fig. 1). Among them, in this study, we characterized three putative RND pumps with an RLS-like motif in MFP proteins with a relatively high homology to those of *V. cholerae* and *E. coli*.

Effects of putative *vexAB*, *vexCD*, or *acrAB1* deletion on susceptibility of *V. vulnificus* to antibacterial compounds

In order to examine whether these pump homologs were involved in the resistance of *V. vulnificus* to antibacterial drugs and other toxic compounds, we constructed *V. vulnificus* strains with a deletion of putative *vexAB* (VVMO6_00057 and VVMO6_00056), *vexCD* (VVMO6_03480 and VVMO6_03481), or *acrAB1* (VVMO6_03994 and VVMO6_

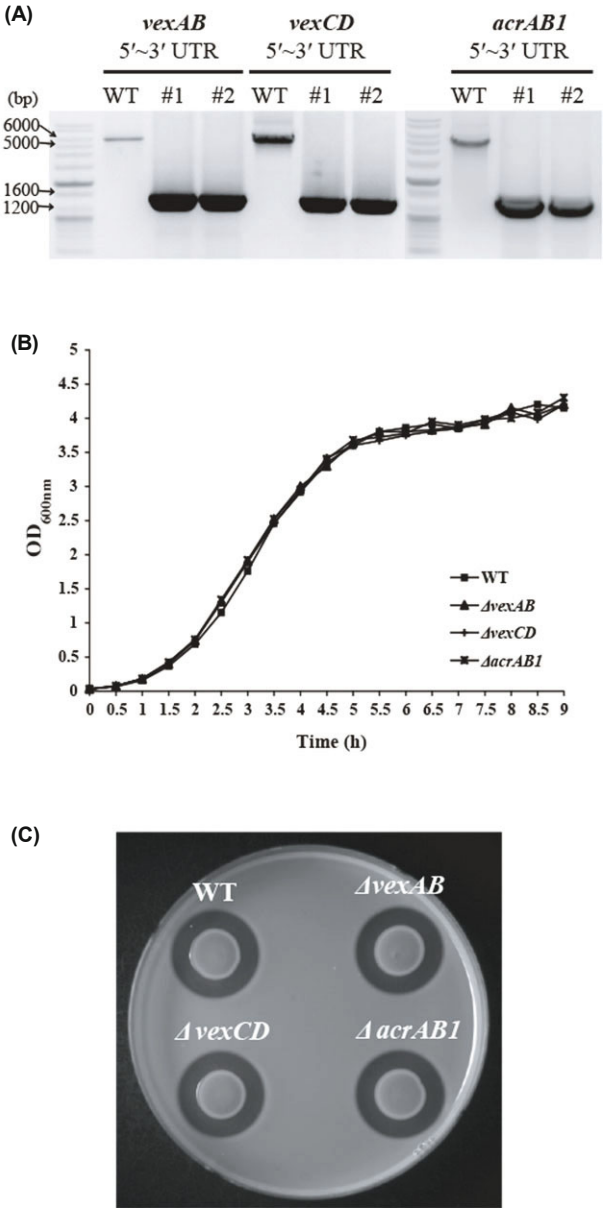

Fig. 2. Characterization of *vexAB*-, *vexCD*-, or *acrAB1*-deleted *V. vulnificus* strains. (A) Verification of *vexAB*, *vexCD*, or *acrAB1* deletions in *V. vulnificus* by PCR. PCR products amplified from four different *V. vulnificus* chromosomes purified from wild-type (WT) and *vexAB*-, *vexCD*-, or *acrAB1*-deleted strains. The expected lengths of DNA fragments amplified from WT and mutants are approximately 5–6 kb and 1.5 kb, respectively. (B) Growth rates of WT *V. vulnificus* and *vexAB*-, *vexCD*-, or *acrAB1*-deleted strains. *V. vulnificus* strains were grown in LBS medium and the growth was monitored by measuring OD_{600} at 30 min intervals. (C) Effects of *vexAB*, *vexCD*, or *acrAB1* deletion on total protease activity of *V. vulnificus*. *V. vulnificus* cells were spotted on a 1.5% skim milk-agar plate, and the cleared zones around bacterial colonies were monitored after 16 h of incubation at 30°C.

03993) genes. To confirm deletion, DNA segments encompassing the targeted genes were PCR-amplified (Fig. 2A). Next, these strains were tested for susceptibility to various antibacterial agents. The results showed that deletion of putative *vexAB* genes rendered *V. vulnificus* cells more susceptible to bile acid, acriflavine, ethidium bromide, and erythromycin (Table 3). In contrast, deletion of putative *acrAB1* genes resulted in moderately increased susceptibility of *V. vulnificus* cells to acriflavine, while deletion of putative *vexCD* had no effect on the susceptibility of *V. vulnificus* to these chemicals. Notably, the deletion of putative *vexAB* genes resulted in dramatically increased susceptibility of *V. vulnificus* cells to erythromycin. Deletion of these genes did not affect the susceptibility of *V. vulnificus* cells to other chemicals, including sodium dodecyl sulfate (SDS) and novobiocin, which are known substrates of other tripartite drug efflux pumps. The degrees of resistance to various antimicrobial chemicals that we observed did not stem from a difference in proliferation, as wild-type and *vexAB*, *vexCD*, or *acrAB1*-deleted cells showed comparable growth rates (Fig. 2B).

Table 3. *In vivo* effects of *vexAB*, *vexCD*, or *acrAB1* deletion on susceptibility of *V. vulnificus*[a] to antibacterial agents

	MIC (μg/ml)			
	WT	Δ*vexAB*	Δ*vexCD*	Δ*acrAB1*
Chemical Detergents				
Bile acid[b]	40	30	40	40
SDS[c]	12,800	12,800	12,800	12,800
Intercalating agents				
Acriflavine[d]	10	8	10	8
EtBr[e]	32	16	32	32
Antibiotics				
Erythromycin[f]	4	0.5	4	4
Novobiocin[g]	0.4	0.4	0.4	0.4

[a] *V. vulnificus* strains MO6-24/O wild-type (WT), Δ*acrAB1*, Δ*vexAB*, and Δ*vexAB* were used.
[b-h] The following concentrations of chemicals were used to measure minimum inhibitory concentrations (MICs).
[b] Bile acid: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/ml
[c] SDS: 0, 12.5, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400, and 12,800 μg/ml
[d] Acriflavine: 0, 2, 4, 8, 10, 12, 14, and 16 μg/ml
[e] EtBr: 0, 1, 2, 4, 8, 16, 32, 64 and 128 μg/ml
[f] Erythromycin: 0, 0.1, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 ng/ml
[g] Novobiocin: 0, 0.1, 0.2, 0.3, 0.4, and 0.5 μg/ml
The experiments were repeated three times.

Effects of *vexAB*, *vexCD*, or *acrAB1* deletion on total protease activity of *V. vulnificus*

A previous report indicated a functional role for the secretion pump proteins in the protease activity of *Pseudomonas fluorescens* (Son *et al*., 2012). For this reason, we examined the effects of *vexAB*, *vexCD*, or *acrAB1* deletion on the protease activity of *V. vulnificus*. The results showed that the *vexAB, vexCD*, or *acrAB1* deletion mutants had no significant changes in protease activity compared to wild-type cells (Fig. 2C).

Effects of antibacterial chemicals on expression of RND protein-encoding genes

We investigated the regulation of the RND protein-encoding genes upon treatment with antibacterial chemicals that were associated with *vexAB*, *vexCD*, or *acrAB1* deletion mutants. Semi-quantitative RT-PCR was performed with total RNA from wild-type cells grown in LBS medium in the presence of bile acid (10 and 20 μg/ml), acriflavine (1 and 2 μg/ml), erythromycin (0.5 and 1 μg/ml), or with no addi-

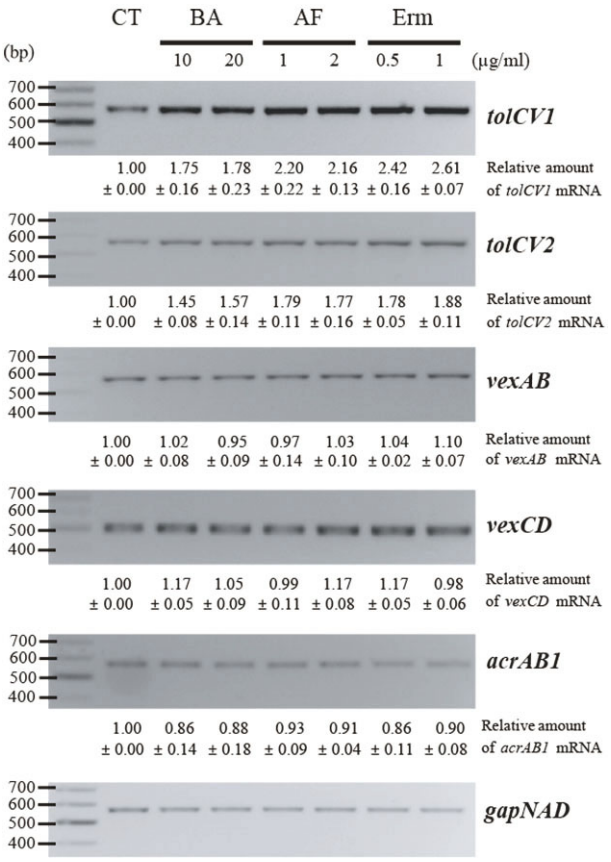

Fig. 3. Effects of antibacterial chemicals on expression of the RND pump- encoding genes Steady-state levels of each mRNA were assessed using semi- quantitative RT-PCR (CT, control; BA, bile acid; AF, acriflavine; and Erm, erythromycin). The relative amounts of mRNA transcripts were measured by setting the amount of each mRNA in wild-type *V. vulnificus* cells grown in LBS medium in the absence of additional chemicals to 1. The abundance of *gapNAD* was measured as an internal standard to evaluate the total amount of RNA in each reaction. The experiments were repeated three times and averaged. The error bars (standard errors of the mean) were used to indicate the range of assay results.

tional chemicals. We observed that the steady-state mRNA levels of *tolCV1* and *tolCV2* were increased by approximately 1.5–2.6 fold in the presence of all of these chemicals. However, the expression levels of *vexAB*, *vexCD*, and *acrAB1* mRNA were not significantly affected upon exposure to these chemicals (Fig. 3).

Discussion

Our study shows that the *V. vulnificus* homologs of VexAB largely contribute to *in vitro* antimicrobial resistance with a broad substrate specificity that was partially redundant with the AcrAB pump homolog. We were not able to ascribe a function to the homolog of the VexCD efflux system. This result does not exclude the possibility that this efflux system may function in antimicrobial resistance under conditions different than those used in this study.

The broad substrate specificity of the putative VexAB efflux system and the degree of increase in the susceptibility of *vexAB*-deleted cells to various antimicrobial chemicals suggest that this efflux system may utilize TolCV1 as an OMF. Deletion of *tolCV1* has previously been shown to render *V. vulnificus* more susceptible to chemical detergents, DNA intercalating agents, and antibiotics (Lee *et al.*, 2014). Considering that TolCV2 plays a limited role in *V. vulnificus* resistance to erythromycin, novobiocin, and tetracycline (Lee *et al.*, 2014), it is unlikely that the putative VexAB and AcrAB efflux systems use TolCV2 as an OMF.

Unlike *V. cholerae vexAB* and *vexCD* (Bina *et al.*, 2006), we were unable to detect induced expression of putative *vexAB*, *vexCD*, and *acrAB1* genes in response to acriflavine, bile acid, or erythromycin. In contrast, the expression levels of *tolCV1* and *tolCV2* were 1.5–2.6 times higher in cells exposed to these chemicals than in untreated cells. These results indicate that *V. vulnificus* has an expression mechanism for these RND efflux systems that is different from *V. cholerae*.

RND efflux systems contribute to the development of multidrug resistance in other pathogenic bacteria (Webber and Piddock, 2003; Nishino *et al.*, 2006). Our study provides the first step toward understanding the functional role of RND efflux pumps in the antimicrobial resistance of *V. vulnificus*.

Acknowledgements

This research was supported by an NRF Grant (2013R1A1A-2006953) funded by the Ministry of Education, Science, and Technology, Republic of Korea, and the Chung-Ang University Research Scholarship Grants in 2012.

References

Bavro, V.N., Pietras, Z., Furnham, N., Pérez-Cano, L., Fernández-Recio, J., Pei, X.Y., Misra, R., and Luisi, B. 2008. Assembly and channel opening in a bacterial drug efflux machine. *Mol. Cell.* **30**, 114–121.

Bina, J.E., Alm, R.A., Uria-Nickelsen, M., Thomas, S.R., Trust, T.J., and Hancock, R.E. 2000. *Helicobacter pylori* uptake and efflux: basis for intrinsic susceptibility to antibiotics *in vitro*. *Antimicrob. Agents Chemother.* **44**, 248–254.

Bina, J.E., Provenzano, D., Wang, C., Bina, X.R., and Mekalanos, J.J. 2006. Characterization of the *Vibrio cholerae* vexAB and vexCD efflux systems. *Arch. Microbiol.* **186**, 171–181.

Du, D., Wang, Z., James, N.R., Voss, J.E., Klimont, E., Ohene-Agyei, T., Venter, H., Chiu, W., and Luisi, B.F. 2014. Structure of the AcrAB-TolC multidrug efflux pump. *Nature* **509**, 512–515.

Hwang, W., Lee, N.Y., Kim, J., Lee, M.A., Kim, K.S., Lee, K.H., and Park, S.J. 2011. Functional characterization of EpsC, a component of the Type II secretion system, in the pathogenicity of *Vibrio vulnificus*. *Infect. Immun.* **79**, 4068–4080.

Kawano, H., Miyamoto, K., Yasunobe, M., Murata, M., Myojin, T., Tsuchiya, T., Tanabe, T., Funahashi, T., Sato, T., Azuma, T., *et al.* 2014. The RND protein is involved in the vulnibactin export system in *Vibrio vulnificus* M2799. *Microb. Pathog.* **75**, 59–67.

Kim, H.M., Xu, Y., Lee, M., Piao, S., Sim, S.H., Ha, N.C., and Lee, K. 2010. Functional relationships between the AcrA hairpin tip region and the TolC aperture tip region for the formation of the bacterial tripartite efflux pump AcrAB-TolC. *J. Bacteriol.* **192**, 4498–4503.

Kim, I.H., Wen, Y., Son, J.S., Lee, K.H., and Kim, K.S. 2013. The fur-iron complex modulates expression of the quorum-sensing mas-

ter regulator, SmcR, to control expression of virulence factors in *Vibrio vulnificus*. *Infect. Immun*. **81**, 2888–2898.

Kohler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L.K., and Pechère, J.C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol. Microbiol*. **23**, 345–354.

Koronakis, V., Eswaran, J., and Hughes, C. 2004. Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu. Rev. Biochem*. **73**, 467–489.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. 2007. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol.* **73**, 777–784.

Lee, M., Jun, S.Y., Yoon, B.Y., Song, S., Lee, K., and Ha, N.C. 2012. Membrane fusion proteins of type I secretion system and tripartite efflux pumps share a binding motif for TolC in Gram-negative bacteria. *PLoS One* **7**, e40460.

Lee, M., Kim, H.L., Song, S., Joo, M., Lee, S., Kim, D., Hahn, Y., Ha, N.C., and Lee, K. 2013. The α-barrel tip region of *Escherichia coli* TolC homologs of *Vibrio vulnificus* interacts with the MacA protein to form the functional macrolide-specific efflux pump MacAB-TolC. *J. Microbiol.* **51**, 154–159.

Lee, S., Song, S., Lee, M., Hwang, S., Kim, J.S., Ha, N.C., and Lee, K. 2014. Interaction between the α-barrel tip of *Vibrio vulnificus* TolC homologs and AcrA implies the adapter bridging model. *J. Microbiol.* **52**, 148–153.

Li, X.Z., Nikaido, H., and Poole, K. 1995. Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. **39**, 1948–1953.

Lim, B., Ahn, S., Sim, M., and Lee, K. 2014. RNase III controls *mltD* mRNA degradation in *Escherichia coli*. *Curr. Microbiol*. **68**, 518–523.

Misra, R. and Bavro, V.N. 2009. Assembly and transport mechanism of tripartite drug efflux systems. *Biochim. Biophys. Acta.* **1794**, 817–825.

Nishino, K., Latifi, T., and Groisman, E.A. 2006. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol*. **59**, 126–141.

Park, J., Cho, Y., Chun, J., Seok, Y., Lee, J., Kim, K., Lee, K., Park, S., and Choi, S. 2011. Complete genome sequence of *Vibrio vulnificus* MO6-24/O. *J. Bacteriol*. **193**, 2062–2063.

Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D.E., and Bianco, N. 1996. Expression of the multidrug resistance operon mexA-mexB-oprM in *Pseudomonas aeruginosa*: mexR encodes a regulator of operon expression. *Antimicrob. Agents Chemother*. **40**, 2021–2028.

Rahman, M.M., Matsuo, T., Ogawa, W., Koterasawa, M., Kuroda, T., and Tsuchiya, T. 2007. Molecular cloning and characterization of all RND-type efflux transporters in *Vibrio cholerae* non-O1. *Microbiol. Immunol.* **51**, 1061–1070.

Son, M., Moon, Y., Oh, M.J., Han, S.B., Park, K.H., Kim, J.G., and Ahn, J.H. 2012. Lipase and protease double-deletion mutant of *Pseudomonas fluorescens* suitable for extracellular protein production. *Appl. Environ. Microbiol*. **78**, 8454–8462.

Song, S., Hwang, S., Lee, S., Ha, N.C., and Lee, K. 2014. Interaction mediated by the putative tip regions of MdsA and MdsC in the formation of a Salmonella-specific tripartite efflux pump. *PLoS One* **9**, e100881.

Symmons, M.F., Bokma, E., Koronakis, E., Hughes, C., and Koronakis, V. 2009. The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proc. Natl. Acad. Sci. USA* **106**, 7173–7178.

Tamura, N., Murakami, S., Oyama, Y., Ishiguro, M., and Yamaguchi, A. 2005. Direct interaction of multidrug efflux transporter AcrB and outer membrane channel TolC detected via site-directed disulfide cross-linking. *Biochemistry* **44**, 11115–11121.

Val, M.E., Kennedy, S.P., El Karoui, M., Bonne, L., Chevalier, F., and Barre, F.X. 2008. FtsK-dependent dimer resolution on multiple chromosomes in the pathogen *Vibrio cholerae*. *PLoS Genet*. **4**, e1000201.

Webber, M.A. and Piddock, L.J. 2003. The importance of efflux pumps in bacterial antibiotic resistance. *J. Antimicrob. Chemother*. **51**, 9–11.

Wright, A.C., Morris, J.G. Jr., Maneval, D.R. Jr., Richardson, K., and Kaper, J.B. 1985. Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. *Infect. Immun*. **50**, 922–924.

Xu, Y., Lee, M., Moeller, A., Song, S., Yoon, B.Y., Kim, H.M., Jun, S.Y., Lee, K., and Ha, N.C. 2011a. Funnel-like hexameric assembly of the periplasmic adapter protein in the tripartite multidrug efflux pump in Gram-negative bacteria. *J. Biol. Chem*. **286**, 17910–17920.

Xu, Y., Song, S., Moeller, A., Kim, N., Piao, S., Sim, S.H., Kang, M., Yu, W., Cho, H.S., Chang, I., *et al.* 2011b. Functional implications of an intermeshing cogwheel-like interaction between TolC and MacA in the action of macrolide-specific efflux pump MacAB-TolC. *J. Biol. Chem*. **286**, 13541–13549.

Zgurskaya, H.I. and Nikaido, H. 1999. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**, 7190–7195.