Properties and Expression of a Multidrug Efflux Pump AcrAB-KocC from *Klebsiella pneumoniae*

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We previously reported that we had cloned genes responsible for multidrug resistance from the chromosomal DNA of *Klebsiella pneumoniae* MGH78578 using a drug-hypersusceptible *Escherichia coli* strain as a host. One of the recombinant plasmids pETV6 conferred resistance to host cells against a wide range of antimicrobial agents, dyes and detergents. It was revealed that this plasmid carried the *acrB_{Kp}* gene and a part of the *acrA_{Kp}* gene coding for a multidrug efflux pump belonging to the RND family. We cloned the whole *acrA_{Kp}B_{Kp}* operon of *K. pneumoniae* and characterized the pump. The AcrAB pump utilized TolC as an outer membrane component in cells of *E. coli*. Elevated energy-dependent efflux of ethidium was observed with cells possessing AcrA_{Kp}B_{Kp}-TolC. We cloned a gene coding for an ortholog of TolC from chromosomal DNA of *K. pneumoniae*, and designated it *kocC*. It seems that the AcrA_{Kp}B_{Kp}-KocC complex functions as a potent multidrug efflux pump in *K. pneumoniae*. We observed a higher level of expression of *acrA_{Kp}* in *K. pneumoniae* MGH78578, a multidrug resistant strain, compared with ATCC10031, a drug susceptible strain.

Key words multidrug efflux pump; AcrAB-KocC; resistance nodulation cell-division; drug resistance; *Klebsiella pneumoniae*

A great many people in the world die of pneumonia every year according to the data by the World Health Organization (<http://www.who.int/whr/2004/annex/en/index.html>). *Klebsiella pneumoniae* is one of the causative agents of bacterial pneumonia. *K. pneumoniae* also causes infections in the respiratory tract, urinary tract and blood stream.^{1,2} Increasing number of *K. pneumoniae* isolates that show resistance against multiple drugs have been reported in the world.³ Thus, it is becoming difficult to treat patients infected with multidrug resistant strains of *K. pneumoniae* by using antibacterial agents.

Multidrug efflux pumps play a major role in multidrug resistance in bacteria by extruding a wide variety of antimicrobial agents, including antibiotics, dyes, antiseptics and detergents. Over-expression of genes for multidrug efflux pumps results in elevated resistance of bacteria against multiple drugs, and is a serious problem in the clinical setting.⁴ There are several types of multidrug efflux pumps in bacteria.⁵ Among the several types of known efflux pumps, the resistance nodulation cell-division (RND)-type efflux pumps play a major role in the multidrug resistance in Gram-negative bacteria.^{6–9}

The AcrAB-TolC pump of *Escherichia coli* is one of the most potent multidrug efflux pumps and is the best characterized RND-type multidrug efflux pump in bacteria.^{10–12} Disruption of the *acrAB* or *tolC* results in great increases in drug susceptibilities in *E. coli*.^{9,13} Therefore it seems that AcrAB-TolC is a principal pump contributing to the intrinsic resistance in *E. coli* against multiple antimicrobial agents.

The RND-type pumps are tripartite, consisting of an inner membrane protein, an outer membrane protein and a periplasmic linker (fusion) protein. Deletion of any one of these three components results in the loss of pump activity.^{9,13,14} It is thought that RND-type efflux pumps utilize the proton-motive force to extrude substrates from cells. Interest-

ingly, several pumps in the RND family extrude β -lactams, which inhibit synthesis of peptidoglycan in the extracellular periplasmic space.^{15,16}

The *acrAB* genes are in an operon in *E. coli*, and their expression is negatively regulated by AcrR.¹⁷ The *acrR* gene is located just upstream of the *acrAB* locus. Another transcriptional regulatory factor, MarA, also regulates the expression of the *acrAB* operon in *E. coli*, and over-expression of *marA* elicits elevated expression of *acrAB* and results in elevated resistance of the cells against multiple antimicrobial agents.¹⁸

Orthologs of AcrAB have been reported in many bacteria of the Enterobacteriaceae family.^{19–23} Domenech-Sanchez *et al.* suggested a relation between quinolone resistance and the AcrAB ortholog in *K. pneumoniae*.²⁴ Mazzariol *et al.* reported that the *acrAB* ortholog has been over-expressed in a clinically isolated quinolone resistant strain of *K. pneumoniae*.²⁵

We previously reported on the cloning of many genes responsible for multidrug resistance from the chromosomal DNA of *K. pneumoniae* MGH78578, a clinically isolated multidrug resistant strain.²⁶ One of the genes responsible for multidrug resistance was *acrB_{Kp}*. To distinguish between *E. coli* AcrAB and its ortholog in *K. pneumoniae*, we describe here the ortholog as AcrA_{Kp}B_{Kp} and their genes *acrA_{Kp}B_{Kp}*. Here we report on the gene cloning and the properties of the AcrA_{Kp}B_{Kp} pump from *K. pneumoniae*, and expression of *acrA_{Kp}* in *K. pneumoniae* cells.

MATERIALS AND METHODS

Bacterial Strains and Growth The bacterial strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* MGH78578 was kindly provided by Dr. Michael McClelland of the Sidney Kimmel Cancer Center in San Diego,

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Table 1. Bacterial Strains and Plasmids Used in This Study

Bacterial strain or plasmid		Reference
<i>K. pneumoniae</i>		
MGH78578	A clinical isolate showing multidrug resistance	
ATCC10031	An ATCC collection	
<i>E. coli</i>		
KAM32	Deletion mutant of <i>acrB</i> and <i>ydhE</i>	19)
KAM33	Deletion mutant of <i>acrAB</i> and <i>ydhE</i>	28)
KAM43	Deletion mutant of <i>acrAB</i> , <i>tolC</i> and <i>ydhE</i>	28)
Plasmids		
pSTV28	Vector	TaKaRa Co.
pETV6	pSTV28-derived recombinant plasmid carrying <i>acrB_{Kp}</i>	This work
pETV62	pSTV28-derived recombinant plasmid carrying <i>acrA_{Kp}B_{Kp}</i>	This work
pKTC44	pSTV28-derived recombinant plasmid carrying <i>kocC</i>	This work
pSET2	pSTV29-derived recombinant plasmid carrying <i>tolC</i>	This work
pBR322	Vector	
pETV63	pBR322-derivatived recombinant plasmid carrying <i>acrA_{Kp}B_{Kp}</i>	This work

CA, U.S.A.. *K. pneumoniae* ATCC10031 was purchased from American Type Culture Collection. *E. coli* KAM32, KAM33 and KAM43 were constructed in our laboratory. Cells were grown in L medium (1% polypeptone, 0.5% yeast extract and 0.5% NaCl, pH 7.0), except for measurement of minimum inhibitory concentration (MIC), at 37 °C under aerobic conditions. Growth of cells was monitored turbidimetrically at 650 nm.

Gene Disruption in *E. coli* Gene disruption was performed in *E. coli* by the method of Datsenko *et al.*²⁷⁾ The details of disruption of *acrAB* and *tolC* in *E. coli* were described previously.²⁸⁾

Cloning of *acrB_{Kp}* and *acrA_{Kp}* We previously reported the cloning of genes responsible for multidrug resistance from the chromosomal DNA of *K. pneumoniae* MGH78578.²⁶⁾ A recombinant plasmid, pETV6, is one of the candidate plasmids. We found that pETV6 carries *acrB_{Kp}* and a part of *acrA_{Kp}*. A plasmid pETV62 carrying whole *acrA_{Kp}B_{Kp}* was constructed by the following method. Primers for amplification of *acrA_{Kp}* were *acrAKP-F* and *acrAKP-R* (Table 2). Polymerase Chain Reaction (PCR) was performed with a PTC-100TM Programmable Thermal Controller (MJ Research, Inc.). A 0.94-kbp PCR product containing the whole *acrA_{Kp}* gene and a part of *acrB_{Kp}* was generated using chromosomal DNA from *K. pneumoniae* MGH78578 as template under the following PCR conditions: initial denaturation of 2 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 51 °C, 1 min at 68 °C. The amplified PCR fragment was digested with endonucleases *SalI* and *AflII*, and ligated with pETV6 digested with the same restriction enzymes. Cells of *E. coli* KAM33 lacking *acrAB* were transformed with the resulting plasmid, and the transformants were selected on L agar plates containing 20 µg/mg chloramphenicol. The resulting plasmid is pETV62. Another plasmid pETV63 was constructed as follows. pETV62 was digested with *SmaI* and *SphI*, and resulting DNA fragment with 4.7-kbp was ligated to pBR322 digested with *EcoRV* and *SphI*. pETV62 is a pSTV28-based plasmid carrying *acrA_{Kp}B_{Kp}*, and pETV63 is a pBR322-based plasmid carrying *acrA_{Kp}B_{Kp}*.

Cloning of *kocC* Gene of *K. pneumoniae* The *kocC* gene was amplified from chromosomal DNA of *K. pneumoniae* MGH78578 using KOD Plus DNA polymerase (Toyobo

Table 2. Primers Used in This Study

Primer name	Primer sequence
<i>acrAKP-F</i>	5'-GCTGTCGACGGTTAATGACTTTACAGAGG-3'
<i>acrAKP-R</i>	5'-ACATCCGAGAATTCCAGCGT-3'
<i>kocC</i> primer 2 (<i>ScaI</i>)	5'-CGCTAAAGTACTCACAACAAGGAATGCAAATG-3'
<i>kocC</i> primer 3 (<i>EcoT22I</i>)	5'-CATCACAGATGCATAATTACTGACGGAACG-3'
<i>tolC</i> forward primer	5'-TTTGTGTCGACATCGCGCTAAATACTGCTTC-3'
<i>tolC</i> reverse primer	5'-CCCGAATTCGAAGCCCCGTCGTCGT-3'
<i>acrA-F</i> (RT)	5'-GTCCTCAGGTCAGTGGCATT-3'
<i>acrA-R</i> (RT)	5'-ATTGCTCTGCTGCGCCGTT-3'
<i>kocC-F</i> (RT)	5'-TCAGGTTTACCAGCAGGCAC-3'
<i>kocC-R</i> (RT)	5'-GCGCGCCATTTGACATATC-3'
<i>marA</i> Fw	5'-GTGGATCGAGGATAACCTGG-3'
<i>marA</i> Re	5'-TACTCTGCTTGAGCTTCTGC-3'
<i>ramA</i> Fw	5'-TTGATACTATCGTCGAGTGG-3'
<i>ramA</i> Re	5'-CTTCCTTTTCGCGAATATAGC-3'
<i>uncB</i> Fw	5'-AGCGTTGCTAAGAAAAGCGAC-3'
<i>uncB</i> Re	5'-AGGAAGACCCAGACGAACAC-3'

Co.) for 35 reaction cycles, with each cycle consisting of 15 s at 94 °C, 2 min 30 s at 68 °C. The primers used were *kocC* primer 2 (*ScaI*), containing a *ScaI* restriction site, and *kocC* primer 3 (*EcoT22I*), containing an *EcoT22I* restriction site (Table 2). The amplified DNA fragment containing the *kocC* gene was ligated to a vector plasmid pSTV28 digested with *ScaI* and *PstI*. The resulting recombinant plasmid carrying the *kocC* gene is pKTC44.

Cloning of *tolC* Gene of *E. coli* The *tolC* gene was amplified without its promoter region by using the *tolC* forward primer and the *tolC* reverse primer (Table 2) using chromosomal DNA of *E. coli* TG1 as the template. The length of the PCR product was confirmed by 1% agarose gel electrophoresis, and the product was purified by GENECLEAN II kit (Qbiogene). The DNA fragment was digested with *EcoRI*, and then ligated with a vector pSTV29 digested with *SmaI* and *EcoRI*. The resulting plasmid is pSET2. The nucleotide sequence of the insert region in pSET2 was determined by the dideoxy chain termination method. We found no error in the sequence.

Determination of MIC The MICs of various antimicrobial agents were determined in Mueller–Hinton broth (Difco, Sparks, U.S.A.) by the two-fold dilution method.²⁹⁾ Cells in the test medium (10^5 cells/ml) were incubated at 37 °C for 24 h, and the growth was subsequently judged. We measured the MICs three times and obtained the same results.

Ethidium Transport Assay The assay was performed as described previously³⁰⁾ at 37 °C. Ethidium bromide was added to cell suspension at a final concentration of 10 μ M.

RNA Preparation and Reverse Transcriptase (RT)-PCR Analysis RNA was isolated from cells harvested at the exponential phase of growth by using an RNeasy mini kit (QIAGEN). Cell lysate was treated with the QIA shredder column before the RNeasy column and treated with RNase-Free DNase (Qiagen Inc., U.S.A.) according to the manufacturer's protocol.

Quantitative real-time RT-PCR was performed with Real Time One Step RNA PCR Kit (TaKaRa Bio Inc.) using the following pairs of oligonucleotides (Table 2); *acrA*-F(RT) and *acrA*-R(RT) for *acrA*, *marA* Fw and *marA* Re for *marA* and *ramA* Fw and *ramA* Re for *ramA*. *uncB* Fw and *uncB* Re for *uncB* were used as the internal control for normalization. A 10 ng of sample of total RNA from *K. pneumoniae* cells was used as a template for one reaction.

RESULTS AND DISCUSSION

Gene Cloning We previously cloned many genes responsible for multidrug resistance from chromosomal DNA of *K. pneumoniae* MGH78578.²⁶⁾ One of the candidate plasmids, pETV6, conferred elevated resistance against ethidium bromide, erythromycin, Rhodamine 6G and so on to drug-hypersusceptible *E. coli* KAM32. Since the genome sequence of the *K. pneumoniae* MGH78578 strain was available (website; <http://genome.wustl.edu/genome.cgi?GENOME=Klebsiella%20pneumoniae>), we partially sequenced the DNA insert of pETV6 in order to identify the open reading frame(s) (ORFs) responsible for the observed elevated drug resistances. We found that the plasmid pETV6 carries one complete ORF and a part of another ORF (Fig.

1). We found that the complete ORF corresponded to *acrB*, and truncated one corresponded to *acrA* (GenBank, ACCSSION No. AJ318073).

We measured the MICs of various antimicrobial agents with *E. coli* KAM32 carrying or not carrying pETV6. Much higher MIC values were observed with multiple antimicrobial agents including cloxacillin, erythromycin, benzalkonium chloride, ethidium bromide, etc., in *E. coli* KAM32/pETV6 compared with *E. coli* KAM32/pSTV28 (control) (Table 3). On the other hand, no change in MICs was detected with ampicillin and kanamycin.

The host *E. coli* KAM32 lacks *acrB* but possesses *acrA*.¹⁹⁾ The plasmid pETV6 possesses the whole *acrB_{Kp}* gene. Thus, it seemed that *AcrB_{Kp}* and *AcrA* formed a tripartite complex together with an outer membrane protein, perhaps TolC, of *E. coli*. We tried to clone *acrA_{Kp}*. In fact, we cloned *acrA_{Kp}* and the promoter region from chromosomal DNA of *K. pneumoniae* MGH78578 by the PCR method, and constructed pETV62 which carries the complete *acrA_{Kp}B_{Kp}* element and its promoter (Fig. 1). We introduced pETV62 into cells of *E. coli* KAM33 which lacks *acrAB*. We observed elevated MICs of cloxacillin, erythromycin, benzalkonium chloride, ethidium bromide and so on with *E. coli* KAM33/pETV62 compared with KAM33/pSTV28 (control)

Fig. 1. Regions of *acrA_{Kp}B_{Kp}* Carried by Recombinant Plasmids

The chromosomal DNA region of *K. pneumoniae* MGH78578 including *acrA_{Kp}B_{Kp}* is shown at the top. Recombinant plasmid pETV6 carries the whole *acrB_{Kp}* gene and a part of *acrA_{Kp}*. The recombinant plasmid pETV62 carries the whole *acrA_{Kp}B_{Kp}* region. The additional region present in pETV62 was cloned by the PCR method. The DNA sequence of the PCR-cloned region was confirmed by sequencing.

Table 3. Drug Susceptibility in *E. coli* Harboring Each Plasmid

Antimicrobial agent	MIC (μ g/ml)			
	KAM32/pSTV28	KAM32/pETV6	KAM33/pSTV28	KAM33/pETV62
Ampicillin	2	2	2	4
Cefotaxime	0.016	0.031	0.031	0.062
Cloxacillin	2	256	2	512
Oxacillin	2	128	1	512
Norfloxacin	0.031	0.062	0.031	0.125
Ofloxacin	0.031	0.062	0.031	0.25
Erythromycin	2	32	4	512
Kanamycin	1	1	1	1
Tetracycline	1	2	1	4
Acriflavine	4	32	4	25
Benzalkonium Cl	16	32	16	32
Ethidium Br	8	256	8	1024
Hoechst 33342	2	16	2	32
Rhodamine 6G	16	2048	8	2048
SDS ^{a)}	256	>2048	256	>2048

Each plasmid carries the following gene; pETV6 (*acrB_{Kp}*), pETV62 (*acrA_{Kp}B_{Kp}*), pSTV28 (vector). a) Sodium dodecyl sulfate.

(Table 3). Likewise, we observed much higher MICs of several antimicrobial agents in *E. coli* KAM33/pETV62 compared with KAM32/pETV6 (Table 3). There are two possibilities for the higher MICs in *E. coli* KAM33/pETV62 than in *E. coli* KAM32/pETV6; 1) AcrB_{Kp} fits well with AcrA_{Kp} than with AcrA, 2) more AcrA_{Kp}B_{Kp} complex is formed since more AcrA_{Kp} is produced from the multi-copy pETV62 plasmid in *E. coli* KAM33/pETV62 compared with AcrA produced from chromosomal *acrA* in *E. coli* KAM32/pETV6. To test the interchangeability between the periplasmic component (AcrA or AcrA_{Kp}) and the inner membrane component (AcrB or AcrB_{Kp}), we constructed a series of plasmids carrying either *acrA_{Kp}-acrB_{Kp}*, *acrA_{Kp}-acrB*, *acrA-acrB_{Kp}* or *acrA-acrB*. These genes were located downstream from *tet* promoter of pBR322. We measured the MICs of various antimicrobial agents with *E. coli* cells harboring each recombinant plasmid. There were no clear differences, however, in the MICs detected (data not shown). Thus, we conclude that there is a very good interchangeability between the components. Therefore, we conclude that the difference in the MICs between *E. coli* KAM32/pETV6 and *E. coli* KAM33/pETV62 is due to the difference in the amount of AcrA_{Kp} produced in the cells, namely the amount of the AcrA_{Kp}B_{Kp} pump. It seems that the outer membrane component TolC is abundant in *E. coli*.

We observed no change in the MICs of any antimicrobial agent with *E. coli* KAM33/pETV6 possessing *acrB_{Kp}* but lacking *acrA*, compared with KAM33/pSTV28 (data not shown). Thus, periplasmic linker (fusion) protein, AcrA_{Kp} or AcrA, is necessary for the function of AcrB_{Kp}.

It should be pointed out that the orientation of *acrB_{Kp}* is the opposite from that of the lactose promoter in plasmid pETV6. There is no original promoter for the *acrA_{Kp}B_{Kp}* operon in pETV6. This means that some sequence in the *acrA_{Kp}* region functions as a promoter. In fact, we found a promoter-like sequence in the *acrA_{Kp}* region (about 460 base pairs upstream from the *acrB_{Kp}* gene) (data not shown).

Ethidium Efflux We tested the efflux activity of AcrA_{Kp}B_{Kp} with *E. coli* KAM33/pETV62 in comparison to that of *E. coli* KAM33/pSTV28 (Fig. 2). We observed a much lower intracellular level of ethidium with *E. coli* KAM33/pETV62 compared with that of *E. coli* KAM33/pSTV28 under energized conditions, indicating strong efflux activity due to AcrA_{Kp}B_{Kp} in *E. coli* KAM33/pETV62. Addition of the H⁺ conductor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) resulted in a higher level of intracellular ethidium (Fig. 2). The same plateau level was observed with the two types of cells after addition of CCCP. These results support the view that AcrA_{Kp}B_{Kp} functions as an energy-dependent efflux pump, perhaps together with an outer membrane component, TolC, in *E. coli* cells.

An Outer Membrane Component for the Pump The RND-type multidrug efflux pumps are tripartite. The plasmid pETV62 carries no gene for an outer membrane protein. It is likely that AcrA_{Kp}B_{Kp} utilizes TolC of *E. coli* as the outer membrane component when functioning as a drug efflux pump in *E. coli* cells. We tested this possibility by introducing pETV63, a pBR322-based plasmid carrying *acrA_{Kp}B_{Kp}* (Table 1), into cells of *E. coli* KAM43 which lacks *tolC* and *acrAB*. We observed no increases in the MICs of erythromycin and others with *E. coli* KAM43/pETV63 compared with

Fig. 2. Energy-Dependent Ethidium Efflux Due to AcrA_{Kp}B_{Kp}

Ethidium efflux in cells was monitored continuously by measuring the fluorescence of ethidium. The assay was performed in the presence of potassium lactate as a respiratory substrate. At the time-point indicated by the first downward arrow, ethidium bromide (EtBr) was added at 10 μ M to the cell suspension. At the time-point indicated by the second arrow, CCCP was added at 100 μ M. A, *E. coli* KAM33/pSTV28 (control); B, *E. coli* KAM33/pETV62.

Table 4. MICs of Several Antimicrobial Agents

Antimicrobial agent	MIC (μ g/ml)				
	<i>E. coli</i> KAM43				
	/pBR322 /pSTV28	/pETV63 /pSTV28	/pETV63 /pSET2	/pBR322 /pKTC44	/pETV63 /pKTC44
Erythromycin	2	2	128	4	128
Acriflavine	2	2	128	2	128
Ethidium Br	2	2	512	4	1024
Kanamycin	1	1	1	1	1

E. coli KAM43 harboring two of the following plasmids were used for MIC testing; pETV63 (carrying *acrA_{Kp}B_{Kp}* genes of *K. pneumoniae*), pKTC44 (carrying *kocC* gene of *K. pneumoniae*), pSET2 (carrying *tolC* gene of *E. coli*), pBR322 (vector) or pSTV28 (vector).

E. coli KAM43/pSTV28 (control) (Table 4). An additional introduction of pSET2, a pSTV28-based plasmid carrying *tolC*, into cells of *E. coli* KAM43/pETV63 resulted in a great increase in the MICs of erythromycin, acriflavine and ethidium bromide (Table 4). Thus, it is clear that TolC of *E. coli* is necessary for AcrA_{Kp}B_{Kp} to function as a multidrug efflux pump in *E. coli* cells.

These results suggest that an outer membrane component is necessary for AcrA_{Kp}B_{Kp} to function as a pump in cells of *K. pneumoniae*. There is no putative gene for an outer membrane component next to the *acrA_{Kp}B_{Kp}* locus in the chromosome of *K. pneumoniae*. Thus, we searched for gene(s) as candidates for ortholog(s) of TolC in the genome sequence of *K. pneumoniae* (<http://genome.wustl.edu/genome.cgi?GENOME=Klebsiella%20pneumoniae>). We found one putative gene for an outer membrane component, the deduced amino acid sequence of which showed high identity (83%) and similarity (98%) with TolC of *E. coli*. We cloned the gene from the chromosomal DNA of *K. pneumoniae* MGH78578 by the PCR method, and designated the gene as *kocC*. The *kocC* gene is located just downstream from the *lac* promoter in a plasmid pKTC44. We tested whether KocC was able to function with AcrA_{Kp}B_{Kp}. Plasmid pETV63 carrying *acrA_{Kp}B_{Kp}* and pKTC44 carrying *kocC* were co-introduced into cells of *E. coli* KAM43 lacking *acrAB* and *tolC*. *E. coli* KAM43/pETV63/pKTC44 showed greatly increased MICs of erythromycin, acriflavine and ethidium bromide (Table 4). Thus, we conclude that AcrA_{Kp}B_{Kp} can utilize KocC as an outer membrane component and can extrude

erythromycin and others from cells. It is highly likely that the AcrA_{Kp}B_{Kp}-KocC tripartite complex functions as a multidrug efflux pump in cells of *K. pneumoniae*. *E. coli* KAM43/pETV63/pSTV28 lacking the outer membrane component showed no increase in the MICs. Cells of *E. coli* KAM43/pETV63/pKTC44 (possessing *acrA*_{Kp}*B*_{Kp} and *kocC*) and *E. coli* KAM43/pETV63/pSET2 (possessing *acrA*_{Kp}*B*_{Kp} and *tolC*) showed almost the same MIC values. Thus, we conclude that TolC from *E. coli* can be completely substituted with KocC from *K. pneumoniae* for the function of the drug efflux pump AcrA_{Kp}B_{Kp} at least in *E. coli* cells.

We detected small but reproducible increases in the MICs of erythromycin and ethidium bromide with *E. coli* KAM43/pKTC44/pBR322 compared with *E. coli* KAM43/pSTV28/pBR322 (Table 4). This suggests that KocC can be utilized as an outer membrane component by some drug efflux pump(s) remaining in *E. coli* KAM43.

The three dimensional (3D) structure of the TolC protein has been reported.³¹⁾ We compared the 3D structure of TolC and that of deduced KocC using a computer software package (Swiss-PdbViewer). The 3D structure of TolC and the deduced 3D structure of KocC were very similar (data not shown). The amino acid sequence of the region of TolC that is the interaction site with AcrB^{11,31,32)} was identical in KocC (data not shown). This supports the view that the interchangeability between TolC and KocC is very high.

We measured mRNA expression of *kocC* by the RT-PCR method, and found that *kocC* was expressed well in cells of *K. pneumoniae* MGH78578. We found a promoter-like sequence in a region just upstream of the *kocC* gene. Its expression level was almost same as that of a house-keeping gene *uncB*, which codes for the β subunit of the H⁺-translocating ATPase (data not shown).

Expression Level of *acrA*_{Kp}*B*_{Kp} in *K. pneumoniae* *K. pneumoniae* MGH78578, a clinically isolated strain, showed higher MICs of many antimicrobial agents as reported previously.²⁶⁾ On the other hand, the MICs observed in a strain obtained from American Type Culture Collection, *K. pneumoniae* ATCC10031, were not high.²⁶⁾ We investigated whether the higher MICs in MGH78578 were due to elevated expression of the *acrA*_{Kp}*B*_{Kp}. Expression of *acrA*_{Kp} in MGH78578 was about 2.2-fold higher than that in ATCC10031 (Fig. 3A). This elevated *acrA*_{Kp} expression may be related to some extent to the elevated MICs of multiple antimicrobial agents in MGH78578. It seems also possible that there are some changes in amino acid sequence between AcrA_{Kp}B_{Kp} from MGH78578 and that from ATCC10031. We are now testing this possibility by sequencing the *acrA*_{Kp}*B*_{Kp} from ATCC10031. It should be noted that the band corresponding to *acrA*_{Kp} was easily detected by RT-PCR analysis even in ATCC10031 cells, indicating that the *acrA*_{Kp}*B*_{Kp} operon is expressing well in cells of *K. pneumoniae* under normal growth conditions. As in the case of *E. coli*, it seems that AcrA_{Kp}B_{Kp} is a principal multidrug efflux pump in *K. pneumoniae* and contributes to intrinsic drug resistance of this microorganism.

Schneiders *et al.* reported that mutation or deletion of *acrR*_{Kp}, a regulatory gene for *acrA*_{Kp}*B*_{Kp}, resulted in increased resistance against fluoroquinolones.³³⁾ There was no deletion in the corresponding region of *acrR*_{Kp} of *K. pneumoniae* MGH78578. We found that there were 7 amino acid re-

Fig. 3. Comparisons of the Relative mRNA Expression Levels of *acrA*_{Kp}, *marA* and *ramA* between *K. pneumoniae* ATCC10031 and MGH78578

The expression was determined by the real time RT-PCR method. Relative quantification of the target gene expression was normalized with the mRNA expression of an endogenous reference gene, *uncB*. Panel A; *acrA*_{Kp}, Panel B; *marA*, Panel C; *ramA*. The data represent mean $\pm$ S.D. of four separate experiments. Statistical significance was determined by use of Student's t-test. **p* < 0.05; significantly different from the value of ATCC10031.

Fig. 4. Effect of Salicylic Acid on the Expression of *acrA*_{Kp} and *marA* in *K. pneumoniae* MGH78578

The expression was determined by the real time RT-PCR method. Relative levels of expression of *acrA*_{Kp} (A) and *marA* (B) in comparison with the expression in the control (expression in the absence of salicylic acid) are shown. Salicylic acid (SA) was added at 128 μ g/ml. Salicylic acid at this concentration gave no effect on the growth of *K. pneumoniae* MGH78578. The data represent mean $\pm$ S.D. of four separate experiments. **p* < 0.05; significantly different from the control value.

placements between the primary sequence of AcrR_{Kp} reported by Domenech-Sanchez *et al.*²⁴⁾ (ACCESSION No.AJ318073) and that deduced in MGH78578. Interestingly, all of the 7 replaced residues in AcrR_{Kp} of MGH78578 strain were identical with those in AcrR_{Kp} in one resistant mutant reported by Schneiders *et al.*³³⁾ Thus, AcrR_{Kp} of MGH78578 is a mutant type one reported by them. It is likely that AcrR_{Kp} in MGH78578 is less active as a repressor than that of wild type, and results in elevated expression of the *acrA*_{Kp}*B*_{Kp} operon in MGH78578 than in ATCC10031.

MarA is a positive regulator for the *acrAB* operon in *E. coli*.³⁴⁾ We investigated whether the expression of the *marA* ortholog (*marA*_{Kp}) was more elevated in *K. pneumoniae* MGH78578 than in ATCC10031. We detected about 2.3 times higher expression of *marA*_{Kp} in the MGH strain than in the ATCC strain (Fig. 3B). Thus, MarA may positively regulate the expression of the *acrA*_{Kp}*B*_{Kp} operon in *K. pneumoniae*. It has been reported that the expression of *marA* was greatly enhanced by salicylic acid in *E. coli*.³⁵⁾ We observed about 8-fold increase in the expression of *marA*_{Kp} by adding salicylic acid to growth medium in *K. pneumoniae* (Fig. 4). However, only a small increase in the expression of *acrA*_{Kp} was observed when salicylic acid was added to the growth medium (Fig. 4). Schneiders *et al.* reported that an increase in the expression of *marA*_{Kp} was not observed with a clinically isolated fluoroquinolone-resistant strain of *K. pneumoniae* that also showed elevated resistance against organic solvents.³³⁾ Thus, the effect of *marA*_{Kp} on the regulation of *acrA*_{Kp}*B*_{Kp} expression in *K. pneumoniae* may be different from that in *E. coli*.

It has been reported that the expression of *ramA*, a regula-

tory gene, was enhanced in a multidrug resistant strain of *K. pneumoniae*.³⁶ It has been also reported that expression of *acrA_{Kp}* was elevated in a strain of *K. pneumoniae* in which the expression of *ramA* was increased.³³ RamA is involved in the expression of *acrAB* in *Enterobacter aerogenes* and is closely related to *K. pneumoniae*. RamA of *E. aerogenes* enhances the expression of *marA*, and an increase in RamA expression results in the enhanced expression of *acrAB* even in a *marA*-deleted strain.³⁷ Both RamA and MarA belong to the AraC-XylS transcriptional activator family.^{36,38} It is not clear whether RamA is involved in the regulation of *acrAB* in *K. pneumoniae*. For these reasons, we also measured the expression level of *ramA* (Fig. 3C). The *ramA* expression level in *K. pneumoniae* MGH78578 was about 4-fold lower rather than in *K. pneumoniae* ATCC10031. Thus, it is not clear at the present time whether RamA is involved in the regulation of *acrAB* expression in *K. pneumoniae* or not.

It was reported that the expression of *acrAB* was elevated by induction with several antimicrobial agents in *E. coli*.^{35,39} Morita *et al.* in our laboratory previously reported that expression of *mexCD* of *Pseudomonas aeruginosa* was induced by several antimicrobial agents.⁴⁰ Although we tested the effect of many antimicrobial agents on the expression of *acrA_{Kp}B_{Kp}* in *K. pneumoniae* MGH78578, we observed only a small increase in the level of expression (data not shown). Thus, it seems that *acrA_{Kp}B_{Kp}* is not an inducible operon in *K. pneumoniae* MGH78578.

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