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Putative Negative Regulation of Novel MarB along with MarA upon the Function of AcrAB/TolC Efflux Pump of *Escherichia coli* K-12

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Abstract: This study was focused on the evaluation of MarB alongside with MarA for its regulatory effects upon the efflux function of AcrAB pump, which were induced or not, perhaps as a target. Transductions of *marR* and/or *acrAB* mutation which were derived from Mar and/or AcrAB mutants of wild type *E. coli* K-12, respectively, into the multicopy plasmid in wild type *E. coli* backgrounds or into the chromosome of isogenic parents were done. Minimal inhibitory concentration (MIC) of transduced mutants was compared with their original mutants. This study reports the indirect evidences that suggests a model in which MarB along with MarA have a putative negative regulatory effect upon the efflux function of AcrAB/TolC pump while MarA alone have a positive regulatory effect to the expression of *acrRAB* operon at transcription level. The target of MarB with MarA for its putative negative regulator might be the AcrAB efflux pump. Another efflux system (s) might be negatively regulated by MarB with MarA, and be involved in the efflux of antibiotics which were otherwise extruded preferentially by AcrAB efflux pump.

Key Words: *Escherichia coli*, Multiple-Antibiotic-Resistance (Mar), MarA, MarB, AcrAB Efflux Pump, Mutation, MIC (minimal inhibitory concentration)

INTRODUCTION

AcrAB/TolC efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants[28]. MarA regulates positively its own promoter (*marO/P*) of *marRAB* operon[4,18,20] which is normally repressed by MarR[5,21] in wild type *E. coli.* Also, MarA regulates positively the expression of *acrAB* operon, at transcription level, which encode AcrA and AcrB proteins those are required for AcrAB efflux pump[19,24,26], thereby conferring resistance to a wide variety of antibiotics. TolC is required as a structural protein of AcrAB pump complex of *E. coli*[8]. The function of MarB, however, is not yet elucidated[24,28,36,41].

MarA is one of the members of AraC subfamily of helix-turn-helix transcriptional activator[4,21]. Purified MarA bounds as a monomer to a 16-bp "marbox" located 69 to 54 nucleotides upstream of a putative RNA initiation site of *marRAB* operon[12,20]. Overexpression of

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MarA from multicopy plasmids results in a Mar phenotype even in strains deleted for the *mar* locus[9]. MarA and MarR could regulate the transcription of *marRAB*, *acrAB*, and *soxRS* operon[17,20,30,37]. In *E. coli* Mar mutants, MarA increase the transcription of *micF* whose antisense RNA decrease the expression of OmpF porin[6,7,9,40]. Therefore, both increased efflux of antibiotics by AcrAB pump as a major role and decreased influx, as a minor role, of a wide variety of antibiotics through this porin confers Mar phenotype[19,25,28].

Expression of *acrRAB* operon is modulated by global stress signals, such as antibiotics, organic solvents, redox-stress, decanoate, ethyl alcohol, osmolarity, etc.[2,15,18,25,29,39]. AcrAB efflux pump of *E. coli* extrudes preferentially β-lactams, quinolones (ciprofloxacin, norfloxacin), erythromycin, rifampin, sodium dodecyl sulfate, acriflavine, etc., but could not expel aminoglycoside antibiotics (kanamycin, gentamicin, etc.)[15,24,32,36]. Therefore, AcrAB mutants of *E. coli* is hypersensitive to these toxic substances because of increased accumulation of above toxic agents in the cell[15,24,32,36]. Emr pumps of *E. coli* extrude ethidium bromide and tetraphenylphosphonium[16]. Tetracycline is expelled specially by the TetB pump of *E. coli*[15].

MarB encoded by *marB* gene has yet no known function; however, when *marB* is present on the same DNA sequences with *marA*, it produces a small increase in antibiotic resistance[41]. Increased transcription of *acrAB* is shown in some strains of Mar mutants carrying *marR* mutation[4,18]. This study reports, however, the possible role of MarB with MarA in the putative negative regulation of efflux function of AcrAB/TolC pump as a target. Also, this study reports that another efflux system(s) might be negatively regulated by MarB with MarA, and be involved in the efflux of antibiotics which were otherwise extruded preferentially by AcrAB efflux pump.

MATERIALS AND METHODS

Bacteria, plasmid, and phage

Bacterial strains, plasmids, and phages used in this study as well as their relevant properties are listed in Table 1. Cultures were routinely grown in Luria-Bertani (LB) medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.5% NaCl [Difco, Detroit, Mich.]) or LB agar plate with or without the appropriate antibiotics for selection at 37℃ unless otherwise noted. Also used was lactose-MacConkey agar medium (Difco). Antibiotics or chemical agents were purchased from Sigma (Sigma Co., St. Louis, Mo.): chloramphenicol (Cm), ampicillin (Ap), tetracycline (Tc), ciprofloxacin (Cp), kanamycin (Km), erythromycin (Em), rifampin (Rf), acriflavine (Af), ethidium bromide (Eb), and sodium dodecyl sulfate (SDS). Antibiotics were added to selective media at the following final concentrations except antimicrobial susceptibility test: Ap, 100 μg/ml; Km, 30 μg/ml. Sodium salicylate (SAL) was purchased from Sigma and used as final concentration of 5 mM. Wild type *E. coli* K-12 MC4100 or W3110 was obtained from G. Weinstock or T. Silhavy, respectively. Phage λ*plac*Mu9 (Km^r) or λ467 (Km^r) was obtained from E. Bremer or D. Berg, respectively. Wild type *E. coli* K-12 JM109 and plasmid pUC19 (Lac^+, Ap^r) was purchased from Promega Co. (Madison, Wis).

Mar and/or AcrAB mutant preparation

Mar and/or AcrAB mutant preparation was described in literatures[32,36,42], and modified with the methods of George et al.[10], and McMurry et al.[23], as follows: Wild type *E. coli* MC4100 or W3110 were cultured in 2 ml LB media at 37℃ by shaking (200 r.p.m.) until cultures reached at stationary phase, smeared 0.1 ml of culture on LB agar plates containing Ap, Tc, or Cp at concentrations slightly above the MIC for these agents, incubated at 32℃[30] for 2 to 4 days, and selected colonies as Mar mutants.

Table 1. Bacteria, phage, and plasmid used in this study

Source	Relevant phenotype or genotype	Reference
Escherichia coli		
MC4100	F^- *araD139* Δ *(argF-lac)U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR thi*	G. Weinstock
W3110	F^- LAM^- In *(rrnD-rrnE)1 rph-1*	T. Silhavy
JM109	F^- *endA1* $recA^-$ (rk^-, mk^+) *gyrA96 thi hsdR17 relA1 supE44* λ^- Δ (*lac-proAB*) [F' *traD36 proAB,* $lacI^q$ *lacZ*Δ*M15*]	Promega Co.
MM22	*marR* mutant[a] derived from MC4100 (Ap^{r}[b], MIC = 50 μg/ml)	This study
MM27	*marR* mutant derived from MC4100 (Ap^r, MIC = 200 μg/ml)	This study
AW9	*acrAB* mutant[c] derived from W3110 (Cp^r, MIC = 40 μg/ml)	This study
AM11	*acrAB* mutant derived from MC4100 (Cp^r, MIC = 7 μg/ml)	This study
AW21	*acrAB*:: Tn*5*, *acrAB* mutant derived from W3110	This study
BM4	*marR* and *acrAB* mutant of MC4100 (Tc^r, MIC = 50 μg/ml)	This study
BM20	*acrAB*::Tn*5*, *marR* mutant of MC4100	This study
BM38	*marR* and *acrAB* mutant of MC4100, (Tc^r, MIC = 50 μg/ml)	This study
TMM27	transduced *marR* mutation by λ*plac*Mu9 from MM27 to MC4100	This study
TAM11	transduced *marR* mutation by λ*plac*Mu9 from AM11 to MC4100	This study
TAW21	transduced *acrAB*::Tn*5* by λ*plac*Mu9 from AW21 to MC4100	This study
TBM4	transduced *marR* and *acrAB* mutation by λ*plac*Mu9 from BM4 to MC4100	This study
TBM20	transduced *marR* and *acrAB*::Tn*5* mutationby λ*plac*Mu9 from BM20 to MC4100	This study
TBM38	transduced *marR* and *acrAB* mutation by λ*plac*Mu9 from BM38 to MC4100	This study
Phage		
λ467	λ*b221 rex*::Tn*5*, Km^r, *cI857*, *O*am*29*, *P*am*80*	D. Berg
λ*plac*Mu9	Km^r Mucts*62* ner^+ Ac *'ara'* Mu*S'* *'lacZ lacY imm*λ	E. Bremer
Plasmid		
pUC19	high copy-number vector, Ap^r, $LacZ^+$, $lacI^q$	Promega Co.
pMM22	transduced *marR* mutation from MM22 by λ*plac*Mu9 & subcloned into pUC19	This study
pMM27	transduced *marR* mutation from MM27 by λ*plac*Mu9 & subcloned into pUC19	This study
pAW9	transduced *acrAB* mutation from AW9 by λ*plac*Mu9 & subcloned into pUC19	This study
pAW21	transduced *acrAB*::Tn*5* from AW21 by λ*plac*Mu9 & subcloned into pUC19	This study

[a] The multidrug-resistant phenotypes of these mutants were derived from *marR* mutation in the *marRAB* operon. For details, see text and Fig. 1.

[b] Abbreviations: Ap, ampicillin; Cp, ciprofloxacin; Tc, tetracycline.

[c] The hypersensitive phenotypes to rifampin, acriflavine, ciprofloxacin, and sodium dodecyl sulfate of these mutants were derived from the mutation of *acrA* or *acrB* genes. For details, see text and Fig. 1.

Above steps were repeated on the same media containing higher concentrations of Ap, Tc, or Cp until Mar mutants were selected. These mutants showed the highest level of minimal inhibitory concentration (MIC) of Ap, Tc, or Cp. According to the reports of Ma et al.[18], Sanchez et al.[32], and Sulavik et al.[36], AcrAB mutants were prepared and selected by two methods: i) hypersensitive phenotypes simultaneously against Em, Rf, Af, Eb, and SDS compared with wild type parents, and ii) direct Tn*5* mutagenesis of wild type *E. coli*[34]. *E. coli* harboring two properties of Mar or AcrAB phenotype was selected as Mar and AcrAB mutants.

Tn*5* mutagenesis

The wild type *E. coli* MC4100 or W3100 were infected with λ467 (λ*b221 rex::*Tn*5*, Km^r, *cI857*, *O*am*29*, *P*am*80*) by the methods of Shaw et al.[34]. Wild type *E. coli* were cultured in 1 ml of LB broth with 0.2% maltose (Sigma) at 37℃ for 18 to 24 hours, transfected with λ467 at 1～10 multiplicity of infection (m.o.i.) at 30℃ for 2 hours, smeared after washing on LB agar plates containing 30 μg/ml of Km, incubated at 30℃ for 2 days, and selected Km^r colonies for Tn*5* insertion mutants. To distinguish between spontaneous Km^r mutants and Tn*5* insertional mutants, two cells were grown at the same culture conditions without antibiotics, and cross resistance were examined by antimicrobial susceptibility test with addition of SAL against Km, Cm, Ap, Tc, Cp, Em, Rf, Af, Eb, and SDS[10,28].

UV induction of λ lysogen and transduction of *marR* and/or *acrAB* mutation

UV induction of λ*plac*Mu9 (Km^r) lysogen carrying *marR* and/or *acrAB* mutation was described in literature[3,31]. Also, transduction of *marR* and/or *acrAB* mutation derived from wild type *E. coli* MC4100 or W3110 were described in literature[3,31,35]. Transduction of *marR* and/or *acrAB* mutation was done with λ*plac*Mu9 into the chromosome of wild type MC4100 (*recA*$^+$) via homologous recombination or pUC19 cloning site in wild type *E. coli* JM109 (*recA*$^-$). Detailed methods for UV induction were as follows: Mar and/or AcrAB mutants were cultured in 1 ml LB broth with 0.2% maltose at 32℃ until cells reached at mid-log phase. One hundred μl of Mar and/or AcrAB mutants and 3 μl of λ*plac*Mu9 were mixed, stored 20 min at 37℃ for phage adsorption, poured 0.1 ml on LB agar plate containing 30 μg/ml of Km, and incubated 1 to 2 days at 32℃. Km^r and resistant colonies conferring Mar and/or AcrAB phenotypes were selected, cultured in 1 ml LB broth at 32℃ until cells reached at mid-log phase, and centrifuged at 4,000 g for 10 min. Each pellet was resuspended in 0.5 ml of 10 mM $MgSO_4$ solution, and poured in an sterile, empty petri-dish. UV irradiation (350 erg/mm^2) was carried out on an opened petri-dish. One ml fresh LB broth was added immediately in an UV-irradiated dish, and cultured by shaking (200 r.p.m.) until the cell lysed. After lysis, 1 drop of chloroform was added, and centrifuged at 4,000 g for 10 min. Centrifugations were repeated for washing, supernatants were stored at -70℃. For transduction of *marR* and/or *acrAB* mutation, 3 μl of each UV-induced supernatants were mixed with 300 μl of each recipient strains grown LB broth with 0.2% maltose, stored 20 min at 37℃, and plated on LB agar plates containing appropriate antibiotics at 32℃ for 1 to 2 days. Colonies were selected, confirmed for Mar and/or AcrAB phenotypes by antibiotic susceptibility test, and stored at -70℃ or used for subsequent experiments.

Plasmid preparation and cell transformation

Plasmid mini-preparation or cell transformation treated with $CaCl_2$ were carried out as described in literature[31,35]. Transduced *marR* and/ or *acrAB* mutations by λ*plac*Mu9 into pUC19 cloning site in JM109 were selected by Mar and/or AcrAB phenotype, Lac^-, and Ap^r. Again, transduced strains harboring recombinant pla-

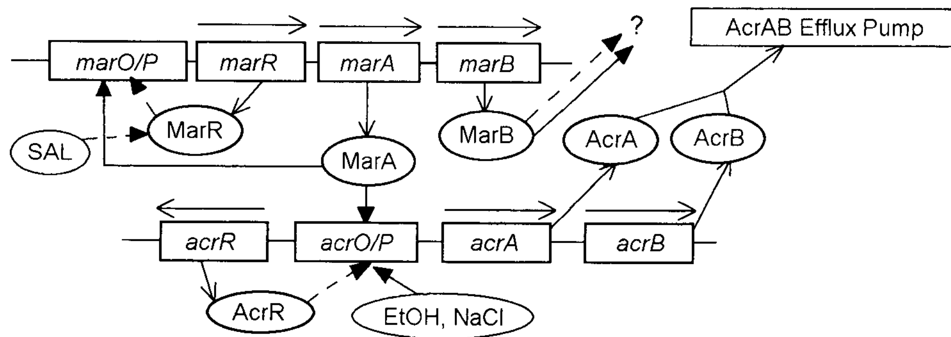

Fig. 1. Regulatory systems involved in the control of multiple-antibiotic-resistance in *Escherichia coli. marRAB* operon (*marO, marP, marR, marA,* and *marB*) and *acrRAB* operon (*acrO, acrP, acrR, acrA,* and *acrB*) are shown above and below, respectively. Arrow heads with line describes as follows: ⟶, the direction of transcription above each gene, or production of protein; ⟶▸, positive regulation to the transcription of target gene; - - ▸, negative regulation to the transcription of target gene. Dark oval circle shows gene product. Pale oval one shows induction condition. SAL, salicylate; EtOH, 4% ethyl alcohol; NaCl, 0.5 M NaCl. Question mark represents the unknown function(s) of MarB.

smids were used for plasmid mini-preparation. Competent, recipient cell for transformation was wild type *E. coli* JM109.

Induction of AcrAB expression

According to report of Ma et al.[18], AcrAB expression was induced by the culture conditions with 4% ethyl alcohol or 0.5M NaCl.

Antibiotic susceptibility test

Agar dilution test was used for determination of MIC of antibiotics against appropriate strains tested in this study. LB agar plates with two-fold serial dilutions (0.01 to 900 μg/ml) of suitable antibiotics were used and interpreted MIC after 20 to 24 hours of incubation at 37℃ by the recommendation of National Committee for Clinical Laboratory Standards (NCCLS)[27]. MIC values were averages of 3 independent plates.

RESULTS

Wild type *E. coli* have many regulatory systems involved in the control of multi-drug resistance (Fig. 1). In *E. coli, marR* mutation increases the expression of *marA* and *marB* genes which encode MarA and MarB, respectively. However, mutation of *acrA* and *acrB* genes which encode AcrA and AcrB structural protein of AcrAB/TolC complex system, respectively, produces possibly defective or absent AcrA and/or AcrB protein (Fig. 1).

For the analysis of the characteristic patterns of antimicrobial resistance of Mar and/or AcrAB mutants, antibiotic susceptibility test was done. Compared with MICs of original wild type *E. coli* MC4100, Mar mutants (MM22, MM27) showed significantly higher levels of MICs of Cm, Ap, Tc, and Cp antibiotics. However, nearly same MICs of Em, Rf, Af, Eb, and SDS were exhibited (Table 2). AcrAB mutants (AW9, AM11, AW21) derived from wild type MC4100 or W3110 showed nearly same or slightly lower levels of MICs of Cm, Ap, and Tc than those of wild type parents. However, MICs of Cp against all AcrAB mutants tested were significantly higher than those of wild type parents. It is well known that Rf, Af, Ap, and SDS antimicrobial agents are preferentially extruded from the cell by AcrAB efflux pump of *E. coli.* Also, in this study, AcrAB mutants showed hypersensitive phenotypes (Table 2). AW21 strain, which was AcrAB mutant prepared from wild type W3110 by Tn*5* mutagenesis, exhibited significantly

Table 2. Comparision of MIC of Mar, AcrAB, and Mar & AcrAB mutants

Strain	MIC[a] (μg/ml) of								
	Cm[b]	Ap	Tc	Cp	Em	Rf	Af	Eb	SDS
MC4100 (W.T.)[c]	5	2.5	3	0.03	400	130	400	600	200
W3110 (W.T.)	5	3	6	0.03	600	15	400	600	300
Mar mutant									
MM22[d]	20	50	30	0.5	400	130	600	600	200
MM27	30	200	15	3	400	130	600	600	200
AcrAB mutant									
AW9	5	3	6	40	0.5	0.5	100	10	2
AM11	5	3	6	7	0.5	0.5	100	10	2
AW21	3	1	1	0.1	0.5	0.1	1	0.5	2
Mar and AcrAB mutant									
BM4	30	20	50	0.5	200	0.5	2	1	2
BM20	20	7	50	0.5	5	0.5	10	1	2
BM38	15	30	50	0.1	400	0.3	1	3	1

a) Determined on LB agar plates containing appopriate antibiotics of two-fold serial dilutions. Results are averages of three plates.
b) Abbreviations: see Materials and Methods.
c) W.T., wild type
d) For detail descriptions, see Table 1.

lower levels of MICs of all antibiotics except Em and SDS than those of AW9 and AM11 mutants, which were AcrAB mutants prepared from spontaneous mutation of wild type parents. Mar and AcrAB mutants showed both properties of Mar or AcrAB mutants in their MIC phenotypes except MIC of Em against BM38 mutants (Table 2).

To evaluate the regulatory effects of MarB with MarA to the efflux function of AcrAB pump, with or without induction of its expression, this study was focused on the MICs of Cp, Ap, Rf, Af, and SDS which were preferentially extruded by AcrAB efflux pump from the cell. Futhermore, this study compared changing differences (x folds) of MICs of transduced strains with those of original mutants (Table 3).

When *marA* and *marB* genes were transduced, with or without induction of AcrAB expression, and expressed as MarA and MarB, *E. coli* TMM27 cells ($MarAB^+$, $AcrAB^-$) and wild type JM109 cells ($MarAB^-$, $AcrAB^+$) harboring pMM22 ($MarAB^+$) or pMM27 ($MarAB^+$) plasmid showed unexpectedly lower MIC levels of Cp, Rf, Af, and SDS in many cases compared with their original Mar parents (data in the gray boxes in Table 3), suggesting that MarB with MarA might have a negative regulatory effect to the efflux function of AcrAB pump, while MarA alone has a positive regulatory effect to the expression of *acrAB* operon. For the analysis of efflux functions of defective or wildtype AcrAB pump, with or without induction and in absence of MarA and MarB, *E. coli* TAM11 ($MarAB^-$, $AcrAB^-$) and TAW-21 ($MarAB^-$, $AcrAB^-$) cells and wild type JM109 cells ($MarAB^-$, $AcrAB^+$) carrying pAW9 ($AcrAB^-$) or pAW21 ($AcrAB^-$) plasmids were evaluated. These cells showed no decrease of

Table 3. Changing differences (x folds) of MICs by MarB with MarA upon function of AcrAB efflux pump with or without the induction of its expression

marA and *marB* location[a]	Induction of AcrAB expression[b]	Relevant phenotype[c]	Strain	Change (x folds)[d] of MIC (μg/ml)							
				Cm	Tc	Eb	Ap	Cp	Rf	Af	SDS
trans	None	$MarAB^{-}$, $AcrAB^{+}$	pUC19/JM109[e]	+1	+1	+1	NT[f]	+1	-5	-5	+2
		$MarAB^{+}$, $AcrAB^{+}$	pMM22/JM109[g]	+1	+6	+2	NT	(+6)	-20	-15	-17
		$MarAB^{+}$, $AcrAB^{+}$	pMM27/JM109	+2	+1	+1	NT	-2	-6	-7	-25
		$MarAB^{-}$, $AcrAB^{+}$	pAW9/JM109	+1	+1	+25	NT	**+1**	**+2**	**+2**	**+1**
		$MarAB^{-}$, $AcrAB^{+}$	pAW21/JM109	+1	+25	+25	NT	**+1**	**+1**	**+15**	**+1**
	EtOH	$MarAB^{-}$, $AcrAB^{+}$	pUC19/JM109	+1	+2	(-5)	NT	-2	-20	-25	-1
		$MarAB^{+}$, $AcrAB^{+}$	pMM22/JM109	+1	+1	(-5)	NT	-2	-25	-25	-30
		$MarAB^{+}$, $AcrAB^{+}$	pMM27/JM109	+1	+5	(-15)	NT	-2	(-5)	(-5)	-30
		$MarAB^{-}$, $AcrAB^{+}$	pAW9/JM109	+1	+20	+7	NT	**+1**	**+1**	**+30**	**+1**
		$MarAB^{-}$, $AcrAB^{+}$	pAW21/JM109	+1	+2	(-6)	NT	(-30)	**+25**	**+2**	(-5)
	NaCl	$MarAB^{-}$, $AcrAB^{+}$	pUC19/JM109	+1	+1	(-15)	NT	+1	-20	-20	+1
		$MarAB^{+}$, $AcrAB^{+}$	pMM22/JM109	+1	+5	(-20)	NT	+1	-20	(-3)	-20
		$MarAB^{+}$, $AcrAB^{+}$	pMM27/JM109	+1	+1	+1	NT	-20	(+2)	(-5)	-5
		$MarAB^{-}$, $AcrAB^{+}$	pAW9/JM109	+1	+1	+1	NT	(-20)	**+2**	**+2**	(-25)
		$MarAB^{-}$, $AcrAB^{+}$	pAW21/JM109	+1	+1	+3	NT	(-15)	**+25**	**+2**	**+30**
cis	None	$MarAB^{+}$, $AcrAB^{+}$	TMM27	+1	+20	+2	(+20)	-2	-2	-5	-20
		$MarAB^{-}$, $AcrAB^{-}$	TAM11	+2	+2	+30	+2	+5	+5	+1	+2
		$MarAB^{-}$, $AcrAB^{-}$	TAW21	+1	+2	+25	+1	+15	+20	+20	+2
		$MarAB^{+}$, $AcrAB^{-}$	TBM4	+1	+1	+25	-20	+30	+7	+1	+1
		$MarAB^{+}$, $AcrAB^{-}$	TBM20	+2	+2	+20	+1	+20	+5	+5	+2
		$MarAB^{+}$, $AcrAB^{-}$	TBM38	+1	+2	+5	-5	+30	+20	+25	+1
	EtOH	$MarAB^{+}$, $AcrAB^{+}$	TMM27	+1	+2	+1	-30	+2	+1	+1	-5
		$MarAB^{-}$, $AcrAB^{-}$	TAM11	+2	+2	+1	+1	+1	+5	+7	+1
		$MarAB^{-}$, $AcrAB^{-}$	TAW21	+1	+1	+15	+2	+2	+30	+25	+20
		$MarAB^{+}$, $AcrAB^{-}$	TBM4	(-5)	+1	+7	+1	+20	+20	+20	-5
		$MarAB^{+}$, $AcrAB^{-}$	TBM20	(-5)	+2	+5	+1	+25	+30	+5	-5
		$MarAB^{+}$, $AcrAB^{-}$	TBM38	+1	+1	+3	-25	+25	+30	+5	+5
	NaCl	$MarAB^{+}$, $AcrAB^{+}$	TMM27	+1	+1	(-5)	+1	+1	-5	-7	-5
		$MarAB^{-}$, $AcrAB^{-}$	TAM11	+2	+1	+1	+1	+2	+1	+1	+1
		$MarAB^{-}$, $AcrAB^{-}$	TAW21	+1	+5	+1	+1	+1	+5	+1	+2
		$MarAB^{+}$, $AcrAB^{-}$	TBM4	+2	+1	+5	+1	+5	=20	+5	+5
		$MarAB^{+}$, $AcrAB^{-}$	TBM20	+1	+1	+7	+2	+15	+7	+5	+5
		$MarAB^{+}$, $AcrAB^{-}$	TBM38	+1	+2	+5	+1	+15	+5	+5	+5

a) Location of *marA* and *marB* relative to the locus of *acrAB* operon
b) See Materials and Methods: EtOH, 4% ethyl alcohol; NaCl, 0.5M NaCl.
c) The sum of relevant phenotypes of *E. coli* host carrying recombinant plasmids or not. If wild type *E. coli* ($AcrAB^{+}$) carries a recombinant plasmids conferring $AcrAB^{-}$ phenotype, this table describes the cell as $AcrAB^{+}$. $MarAB^{-}$ means basal level expression of *marRAB* operon whose expression is normally repressed by MarR in wild type *E. coli* rather than Mar mutants.
d) MIC was determined by three independent antibiotic susceptibility tests. Changing differences (x folds) of MIC levels between test strains and their original Mar and/or AcrAB mutants or wild type backgrounds: +, increase; -, decrease of MIC levels, respectively. Data in the brackets are unexpected or inconsistent results with under that condition. For the data in the gray colored, dark colored, and gray diagonal boxes, see Discussion of this text.
e) Wild type *E. coli* JM109 carrying multicopy pUC19 plasmids. MIC data of pUC19/JM109 shows the changing differences (x folds) of MIC compared with wild type *E. coli* MC4100 or W3110,
f) NT; not tested. Because pUC19 plasmid is ampicillin-resistant (MIC >900 μg/ml).
g) pMM22/JM109 describes wild type *E. coli* JM109 carrying pMM22 plasmids. For recombinant plasmids, see Table 1.

MICs in many cases compared with control such as JM109 carrying pUC19, indicating that there were intact efflux functions of wild type AcrAB pump (data in the dark boxes in Table 3). eHowever remarkably, many MICs of TAM11 (MarAB$^-$, AcrAB$^-$) and TAW21 (MarAB$^-$, AcrAB$^-$), with AcrAB induction and in absence of MarA and MarB, were unexpectedly not decreased compared with original AcrAB mutants (data in above two rows of gray diagonal boxes in Table 3). Interestingly, almost all of the MICs of all transductants (TBM4, TBM20, and TBM38) carrying MarAB$^+$ and AcrAB$^-$ phenotypes, with or without inductions of AcrAB pump, were not decreased compared with original Mar and AcrAB mutants (MarAB$^+$, AcrAB$^-$) (data in gray diagonal boxes in Table 3).

Considering about the data of Cm antibiotics tested, MIC levels were nearly unchanged or stably transduced and expressed in almost all of the 33 test conditions, except 2 cases (data in brackets of Cm column in Table 3), indicating that MarA and MarB were appeared to have no regulatory effects to the Cm efflux through the AcrAB pump. MIC levels correlated with the function of TetB pump were nearly unchanged or increased (data in Tc column of Table 3). The results, that represent the function of Emr efflux pumps of *E. coli* for extrusion of Eb, were, however, highly variable in each test conditions (data in Eb column of Table 3). Changing differences of MICs of Ap, which is extruded mainly by AcrAB pump from the cell, were well correlated with each test conditions, except one case in TMM27 (MarAB$^+$, AcrAB$^+$) (data in bracket of Ap column of Table 3).

There were 249 data from all test conditions shown in Table 3. Among thirty-nine data (100%) which were obtained from the tests for putative negative regulatory effect of MarB along with MarA upon the efflux function of AcrAB pump, thirty-two (82%) (data in gray boxes) were closely related to the test purposes described above, while unexpected data were nine (18%) (data in brackets of related rows of gray boxes). Among twenty-four data (100%) those were tested for the intact efflux function of AcrAB pump, nineteen (79%) (data in dark boxes) were closely related to the intact function while five (21%) were unexpected results (data in brackets of related rows of gray boxes). Among thirty-six data (100%) which were obtained from the test for the target of MarB with MarA and possible existence of another efflux pump(s), thirty (83%) (data in gray diagonal boxes) were closely related to the test purposes while six (17%) were unexpected results.

DISCUSSION

In *Escherichia coli* cell envelope, there are many known efflux pump systems[14,15,24] which are active independently or somewhat related to each other: 1) AcrAB/TolC pump[28], 2) Emr pumps (EmrAB/TolC, EmrD, EmrE)[15,16], 3) TetB pump[15], 4) Bcr pump[15], and 5) homologs of AcrAB or EmrB pump[15,19].

It is well known that there are five important reports concerned about this study: 1) AcrAB/TolC efflux pump plays a major role in the antibiotic resistance phenotype of *E. coli* multiple-antibiotic-resistance (Mar) mutants[28], 2) positive regulatory effect of MarA to the expression of *acrAB* operon thereby conferring Mar phenotype of *E. coli* Mar mutants[17,22,26], 3) AcrAB efflux pump extrude preferentially Em, Cp, Rf, Af, Ap, and SDS[15,18,32], 4) In *E. coli*, *marR* mutation increases the expression of *marA* and *marB* genes which encode MarA and MarB, respectively[1,4,30], and 5) no known function of MarB[4,18,20,24,41].

In this study, *E. coli* Mar mutants (MarAB$^+$, AcrAB$^+$) showed higher MIC levels of Cm, Ap, Tc, and Cp and same MIC levels of Em, Rf, Af, Eb, and SDS compared with their wild type parents, suggesting that there were intact efflux function of wild type AcrAB efflux

pump. These results were closely related to the reports of Okusu et al.[28]. However, AcrAB mutants derived from wild type MC4100 or W3110 showed nearly same or slightly lower levels of MICs of Cm, Ap, and Tc than those of wild type parents. All AcrAB mutants showed hypersensitive phenotypes against antimicrobial agents (such as Em, Rf, Af, Ap, and SDS) which were otherwise extruded mainly from the cell by AcrAB efflux pump of wild type *E. coli*[18,25,32,36]. Exceptionally, MICs of Cp against all AcrAB mutants tested were significantly higher than those of parents. This result remains unclear. Mar and AcrAB mutants showed both properties of Mar or AcrAB mutants in their MIC phenotypes except MIC of Em in case of BM38 mutants.

To evaluate the regulatory effect of MarB along with MarA to the efflux functions of AcrAB pumps, which were induced or not, this study was focused on the MICs of Cp, Ap, Rf, Af, and SDS antibiotics which were preferentially extruded by AcrAB efflux pump from the cell. Futhermore, changing differences of MICs of transduced strains with those of original mutants or wild type backgrounds were compared in this study (Table 3).

When wild type JM109 cells ($MarAB^-$, $AcrAB^+$) harboring pMM22 ($MarAB^+$) or pMM27 ($MarAB^+$) plasmid were tested, these cells showed surprisingly much lower MIC levels of Cp, Rf, Af, and SDS in many cases compared with their original Mar parents (data in the gray boxes in Table 3), suggesting that MarB with MarA might have a negative regulatory effect to the efflux function of AcrAB pump because MarA alone has a positive regulatory effect to the expression of *acrAB* operon.

From the data for the analysis of efflux functions of defective or wild type AcrAB pump, without induction and in absence of MarA and MarB, there were intact efflux functions of wild type AcrAB pump (data in the dark boxes in Table 3). However remarkably, many MICs of TAM11 ($MarAB^-$, $AcrAB^-$) and TAW21 ($MarAB^-$, $AcrAB^-$), with AcrAB induction and in absence of MarA and MarB, were unexpectedly not decreased compared with original AcrAB mutants (data in above two rows of gray diagonal boxes in Table 3), suggesting two possible explanations: 1) according to report of Okusu et al.[28], the presence of a defective proteins of AcrAB pump did not decrease, in part, MIC levels and 2) possible functions of another efflux pump(s)[38] which might be negatively regulated by MarB along with MarA.

In *E. coli*, AcrAB expression is increased by 4% ethyl alcohol and 0.5 M NaCl[18] and by MarA[18,26] whose expression is increased by itself[20] or *marR* mutation[11,13,33]. According to the report of Thanassi et al.[38], accumulation of bile salt such as chenodeoxycholate by the everted membrane vesicles was not decreased by mutations in *acrA* and *emrB* genes and presumably reflects activity of the unknown, another efflux system(s), while intact cells of *E. coli* K-12 appeared to pump out bile salt. Interestingly, same results were obtained in this study. Almost all of the MICs of all transductants (TBM4, TBM20, and TBM38) carrying $MarAB^+$ and $AcrAB^-$ phenotypes, with no induction of AcrAB pump, were not decreased compared with original Mar and AcrAB mutants ($MarAB^+$, $AcrAB^-$) (data in gray diagonal boxes in Table 3), suggesting three possible explanations: 1) another efflux pump(s) might be involved in the efflux of antibiotics which were otherwise preferentially extruded by AcrAB pump. 2) MarB with MarA might be involved in the negative regulation of another efflux pump(s) described above. 3) MarB along with MarA might have a negative regulatory effects to the efflux function of AcrAB pump. Because putative negative regulation of MarB with MarA to the AcrAB target could not be shown possibly in absence or defectiveness of AcrAB pump. Therefore, one of the target(s) of MarB with MarA for its putative negative regulator might be the AcrAB pump.

However, some MICs of TAM11 ($MarAB^-$,

AcrAB⁻) and TAW21 (MarAB⁻, AcrAB⁻), with or without induction of AcrAB pump expression, were not decreased compared with original AcrAB mutants. The possible explanations for these some unexpected results might be one of three points of view: 1) according to the report of Okusu et al.[28], the presence of a defective pump protein did not, in part, cause a hypersensitive phenotype or the influx of antibiotics through leakage, 2) From the report of Sulavik et al.[37], differences of MIC phenotypes in strain backgrounds may play a role, and 3) according to the reports of Thanassi et al.[38] and this study, another efflux system(s) might be involved in the extrusion of antibiotics.

In this study, transduction of Cm resistance was stably transferred, nearly unchanged, and expressed in almost all of the recipient strains. These data suggest that Cm efflux is not regulated by AcrAB pump. TetB efflux pump of *E. coli* for Tc extrusion[15] might play a major role for Tc extrusion, while Emr efflux pumps[16] might play a variable or minor role for Eb extrusion. Okusu et al.[28] indicated that EmrAB efflux pump has little effect on the extrusion of antibiotics.

Analysis for the data significances of this study showed that 82% (data in gray boxes in Table 3) of data suggest the putative negative regulatory effect of MarB with MarA on the efflux function of AcrAB pump, while 79% (data in dark boxes in Table 3) of data were closely related to the well-known, wild type efflux function of AcrAB pump. Therefore, data for the evaluation of regulatory effect of MarB with MarA on the AcrAB pump were very significant. Eight-three percent of data were closely related to the test purposes in which the target of MarB with MarA and possible existence(s) of another efflux pump(s) were included. In this study, it may be argued that background differences in strains may play a role[37] in these test conditions in which transduced mutations into multicopy plasmid were expressed in different *E. coli* strains. However, this possibility is contradicted by the following two data: 1) changing differences of MICs of transductants which were derived from original mutants of different backgrounds were compared with same control group which carried transduced mutations in same backgrounds (data in gray or dark boxes and data in control boxes of Table 3). 2) Results from these test conditions clearly demonstrate the following indirect suggestions. While data from well-known efflux functions of wild type AcrAB pump were appeared as 79% (data in dark boxes of Table 3) of all related test conditions, however, data for the evaluation of putative negative regulatory effect of MarB with MarA on the AcrAB pump were appeared as 82% (data in gray boxes of Table 3) of all related test settings.

In summary, AcrAB efflux pump might be regulated negatively by MarB along with MarA at a functional level of AcrAB pump as a target, while *acrRAB* operon is positively regulated by MarA alone at transcriptional level. Another efflux system(s) might be also regulated by MarB with MarA and involved in the efflux of antibiotics which were otherwise extruded by AcrAB efflux pump.

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=국문초록=

대장균 K-12의 AcrAB/TolC Efflux Pump의 기능에 대한 MarB와 MarA의 추정적 억제조절

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본 연구는 MarA와 함께 MarB가 AcrAB efflux pump를 목표로 하는지 여부, 그리고 항생제의 균체외 배출기능에 대하여 어떤 조절 작용이 있는지를 항생제 내성검사를 통하여 살펴보았다. 본 연구결과는 MarB가 MarA와 함께 AcrAB/TolC efflux pump의 항생제 배출기능을 억제적으로 조절한다는 것을 간접적으로 보여주었으며, 한편 이미 알려진 대로 MarA는 *acrRAB* operon의 발현을 전사 수준에서 positive regulation하므로, MarB는 AcrAB efflux pump의 기능을 억제적으로 조절한다는 것을 암시하고 있다. 그리고 MarA와 함께 MarB 단백질의 작용 목표는 AcrAB efflux pump임을 간접적으로 보여주고 있다. 또한 MarB는 MarA와 함께 대장균의 다른 efflux system(들)의 항생제 배출기능에 대해서도 억제적으로 조절할 가능성이 높은 것으로 나타났다.

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