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# Evidences that Suggest the Spread of Multiple-Antibiotic-Resistance (*mar*) Operon of *Escherichia coli* Mutants among Gram-Negative Bacilli

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**Abstract:** To evaluate the spreading possibilities of the *marRAB* mutation of *E. coli* Mar mutant among gram-negative bacilli, chromosomal *marRAB* mutations of Mar mutants were transduced by λ*plac*Mu9 into pUC19 ($Lac^+$, $Ap^r$) cloning site in another strains of *E. coli* or onto the chrmosome of *S. typhimurium* and *P. aeruginosa*, selected for transduction by Mar phenotype, $Lac^-$, or $Ap^r$, and tested for their antimicrobial resistance with or without addition of salicylate (SAL). Compared with wild type strains of JM109, NM522, harboring pUC19 or not, respectively, all strains of JM109 or NM522 carrying pUC19::*marRAB* mutation showed higher levels of antimicrobial resistance and SAL induction of Mar phenotype than those of wild type. However, in contrast to the original Mar mutants, there were some tendencies of decreased antimicrobial resistance of JM109 or NM522 harboring pUC19::*marRAB* mutation with SAL induction against chloramphenicol (Cm) and tetracycline (Tc), or Tc and ciprofloxacin (Cp), respectively. Almost the same results, as shown as the cases of *E. coli* JM109 or NM522, were obtained from all transductants of *S. typhimurium* and *P. aeruginosa*, except Cp, against which increased antimicrobial resistance with SAL induction was shown. This study, employed the methods of transformation or transduction among intercellular gene transfer methods between gram-negative bacteria, shows the evidences that suggest indirectly the spreading possibilities of *marRAB* mutation among gram-negative bacilli.

**Key Words:** Multiple-Antibiotic-Resistance (Mar), *Escherichia coli*, Mutation

## INTRODUCTION

Multiple-antibiotic-resistance (*marRAB*) operon[1,2,5,12] of *Escherichia coli* is involved in the regulation of intrinsic resistance to many antimicrobials, structurally related or not, or toxic materials[14,15,22,23], the expression of antioxidant genes[1,11], and internal pH homeostasis[30]. The chromosomal *mar* locus is located at 34 min on the *E. coli* genetic map, and consists of *marO* (operator-promoter region), *marR*, *marA*, and *marB* genes[6,33]. In wild type *E. coli*, MarR is expressed constitutively, acts as local repressor[18,19,33] to *mar* promoter region for the transcription of *marA* which mediates the intrinsic resistance to a wide variety of antimicrobials. Therefore, mutation of *marR* exhibits the phe-

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notype of higher levels of antimicrobial resistance than wild type *E. coli*[7]. *marB* has no effect of its own, however, when it is present on the same construct with *marA*, it produces sometimes a small increase in antibiotic resistance[2,33]. Expression of the *E. coli marRAB* operon or *marR* mutation could be induced by a variety of chemical agents including sodium salicylate (SAL), chloramphenicol (Cm), tetracycline (Tc), paraquit etc., and hence these agents could induce the phenotypes of multiple antibiotic resistance[5,19,22].

In *E. coli*, antimicrobial resistance mechanisms by *marR* mutation or SAL-treated cells are associated with the efflux of antibiotics from the cell or toxic substances mostly by partial up-regulation of AcrAB/TolC efflux pump[2,22], or, in part, associated with the decreased intracellular accumulation of a variety of noxious agents by down-regulation of expression of OmpF porin[6,7,9]. *mar*-like DNA sequences or Mar phenotype and their similar functions of antimicrobial resistance were found in the chromosome of *Salmonella typhimurium, Shigella* species, *Pseudomonas aeruginosa, Hemophilus influenzae, Neisseria gonorrhoeae, Mycobacterium*, pneumococci, *Klebsiella pneumoniae, Enterobacter cloacae* etc.[4,8,13,17,26,28]. Among the quinolone resistant *E. coli* isolated from clinical specimens, thirteen percent of *E. coli* were due to the deletion of *marRAB* operon, or point mutation of *marR*[17]. However, EmrAB/TolC efflux pump has little effects to the antimicrobial resistance in clinical isolates of *E. coli*[22,27].

R plasmid, the extrachromosomal genetic element for antimicrobial resistance, is widespread among gram-negative bacilli, is one of the important factors for multiple antibiotic resistance or hospital infections[21,24,29]. This paper reports the evidences that suggest indirectly the spreading possibilities of *marRAB* mutation, related to the multiple antibiotic resistance, among gram-negative bacilli.

## 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 unless otherwise noted. Also used was lactose-MacConkey agar medium (Difco). Antibiotics (Sigma Co., St. Louis, Mo.) were added to selective media at the following final concentrations except antimicrobial susceptibility test: ampicillin (Ap), 100 ug/ml; kanamycin (Km), 30 ug/ml. Sodium salicylate (SAL) was purchased from Sigma and used as final concentration of 5 mM. Phage λ*plac*Mu9 ($Km^r$) was obtained from E. Bremer. *E. coli* JM109 and NM522, and plasmid pUC 19 ($Lac^+$, $Ap^r$) was purchased from Promega Co. (Madison, Wis).

### Mar mutant preparation

Mar mutant preparation was described previously[10], modified with the methods of George et al.[10], and McMurry et al.[20], 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 chloramphenicol (Cm) or Ap at concentrations slightly above the MIC for these agents, incubated at 32℃[30] for 2 to 4 days, and selected colonies as Mar mutants. Above steps were repeated several times on the same media containing higher cencentrations of Cm or Ap, until Mar mutants expressing the highest level of minimal inhibitory concentration (MIC) of Cm or Ap were selected.

### UV induction and transduction

UV induction or transduction was described

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

| Strain | Relevant phenotype or genotype | Reference or source |
|---|---|---|
| *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*⁻ ($r_k^-$, $m_k^+$) *gyrA96 thi hsdR17 relA1 supE44* λ⁻ Δ(*lac-proAB*) [F' *traD36 proAB, lacI*$^q$ *lacZ* Δ *M15*] | Promega Co. |
| NM522 | F⁻ *recA*⁻ Δ(*lac-proAB*) *supE thi hsd5* [F' *traD36 proAB*$^+$ *lacI*$^q$ *lacZ* Δ *M15*] | Promega Co. |
| WCm50[a] | Mar mutant of W3100 ($Cm^{rb}$, MIC = 50 ug/ml) | This study |
| WAp100 | Mar mutant of W3110 ($Ap^r$, MIC = 100 ug/ml) | This study |
| WAp300 | Mar mutant of W3110 ($Ap^r$, MIC = 300 ug/ml) | This study |
| MAp100 | Mar mutant of MC4100 ($Ap^r$, MIC = 100 ug/ml) | This study |
| Gram (−) bacteria | | |
| *S. typhimurium* TT10502 | wild type, Mus*A1* | J. Roth |
| ST41 | transduced *marRAB*[c] mutation by λ*plac*Mu9 from WCm50 into chromosome of *S. typhimurium* TT10502 | This study |
| ST361 | transduced *marRAB* mutation by λ*plac*Mu9 from WAp100 into chromosome of *S. typhimurium* TT10502 | This study |
| *P. aeruginosa* ATCC 27853 | wild type, reference host for antimicrobial susceptibility test | ATCC[d] |
| PA41 | transduced *marRAB* mutation by λ*plac*Mu9 from WCm50 into chromosome of *P. aeruginosa* ATCC 27853 | This study |
| PA361 | transduced *marRAB* mutation by λ*plac*Mu9 from WAp100 into chromosome of *P. aeruginosa* ATCC 27853 | This study |
| Bacteriophage | | |
| 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*$^q$ | Yanisch-Perron |
| pBT41 | pUC19::*marRAB*[e] (from WCm50, $Cm^r$ 50 ug/ml) | This study |
| pBT361 | pUC19::*marRAB* (from WAp100, $Ap^r$ 100 ug/ml) | This study |
| pBT3921 | pUC19::*marRAB* (from MAp100, $Ap^r$ 100 ug/ml) | This study |
| pBT4733 | pUC19::*marRAB* (from WAp300, $Ap^r$ 300 ug/ml) | This study |

[a] Number after each mutant strain name indicates the highest MIC level of corresponding antibiotic.
[b] Abbreviations: Cm, chloramphenicol; Ap, ampicillin
[c] Transduced *marRAB* mutation by λ*plac*Mu9 from WCm50 into chromosome of *S. typhimurium* TT10502, and selected Mar phenotype.
[d] ATCC: American Type Culture Collection
[e] Transduced *marRAB* mutation by λ*plac*Mu9 from WCm50 into pUC19 cloning site, and selected by Mar phenotype, $Lac^-$, and $Ap^r$ in JM109 or NM522 host strain.

in literature[35]. Mar mutants were cultured in 1 ml LB broth with 0.2% maltose at 32℃ until cells reached at mid-log phase. Hundred μl of Mar mutants and 3 μl of λ*plac*Mu9 ($Km^r$) were mixed, stored 20 min at 37℃ for phage adsorption, poured 0.1 ml on LB agar plate containing 30 ug/ml of Km, and incubated 1 to 2 days at 32℃. $Km^r$ and Mar (resistant to specific antibiotics) colonies 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 a sterile, empty petri-dish. UV irradiation (350 erg/$mm^2$) was carried out on an opened petri-dish. One ml of fresh LB broth were added immediately in an irradiated dish, and cultured by shaking (200 r.p.m.) until the cell lysed. After lysis, one drop of chloroform was added, and centrifuged at 4,000 g for 10 min. Centrifugations were repeated, and supernatants were stored at -70℃. Mar transduction was as follows: 3 μl of each UV-induced supernatants with 300 μl of each recipient strains grown LB broth with 0.2% maltose were mixed, stored 20 min at 37℃, and plated on LB agar plates containing appropriate antibiotics at 32℃ for 1 to 2 days. Colonies were selected and stored at -70℃, or used subsequent experiments.

**Plasmid preparation and cell transformation**

Plasmid mini-preparation or cell transformation treated with $CaCl_2$ were carried out as described in literature[31,34]. Transduced DNA sequence containing *marRAB* mutation into pUC 19 cloning site was selected by Mar phenotype, $Lac^-$, and $Ap^r$ in recipient strains for

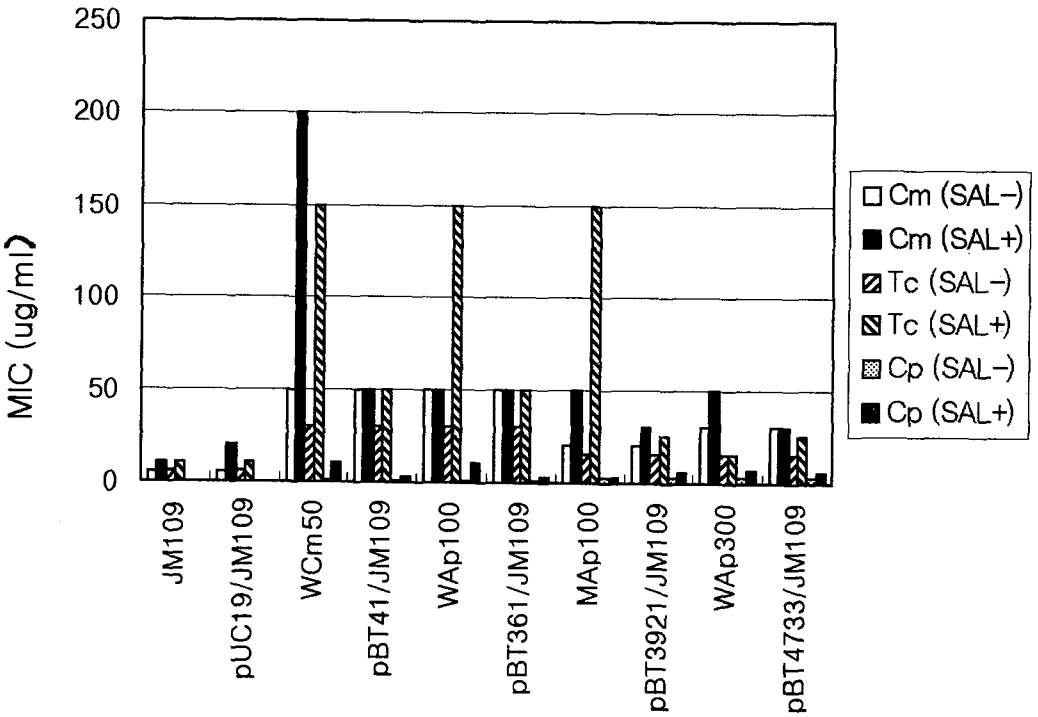


**Fig. 1.** Antimicrobial resistance of transduced *marRAB* mutation by λ*plac*Mu9 from *E. coli* Mar mutants (WCm50, WAp100, MAp100, or WAp300) into pUC19 cloning site (pBT41, pBT361, pBT3921, or pBT4733), respectively. pUC19/JM109 denotes the pUC19 plasmid in JM109 host strain. Ampicillin (Ap) resistant phenotype of each Mar mutants was not tested because of pUC19 ($Ap^r$ MIC = >900 ug/ml). Cm, chloramphenicol; Tc, tetracycline; Cp, ciprofloxacin. SAL+ means the culture with addition of salicylate (SAL); SAL-, without addition of SAL.

transduced plasmid, pUC19::*marRAB* mutation, and used for plasmid mini-preparation or cell transformation. Competent, recipient cells were *E. coli* JM109, or NM522.

### Antibiotic susceptibility test

Agar dilution test was used for determination of MIC of antibiotics against appropriate strains. LB agar plate with two-fold serial dilutions (0.01 to 1,200 ug/ml) of suitable antibiotics was used, and interpreted MIC after 20 to 24 hours of incubation at 37℃ by the recommendation of National Committee for Clinical Laboratory Standards (NCCLS)[25]. MIC values were averages of 3 independent plates.

## RESULTS

### Transducion of *marRAB* mutation and SAL induction in another strains of *E. coli*

Transduced *marRAB* mutation was carried by λ*plac*Mu9 from the chromosome of *E. coli* MC4100 or W3110 Mar mutants (WCm50, WAp100, MAp100, or WAp300) into pUC19 cloning site in *E. coli* JM109 or NM522 host strain, and subsequent selections by Mar phenotype, $Lac^-$, and $Ap^r$ were evaluated for the spreading possibilities of *marRAB* mutation among another strains of *E. coli*, JM109 (Fig. 1) or NM522 (Fig. 2). Also, their antimicrobial resistance and SAL induction of Mar phenotype were evaluated. Compared with wild type strains of JM109 or NM522, harboring pUC19 or not, respectively, all strains of JM 109 or NM522 carrying pUC19::*marRAB* mutation showed significantly higher levels of antimicrobial resistance and SAL induction of Mar phenotype (Fig. 1 and Fig. 2). However, in contrast to the original Mar mutants derived from the wild type *E. coli* MC4100 or W3110, there were some tendencies of decreased antimicrobial resistance of JM109 or NM522 harboring pUC19::*marRAB* mutation with SAL induction, against Cm and Tc (Fig. 1), or Tc and

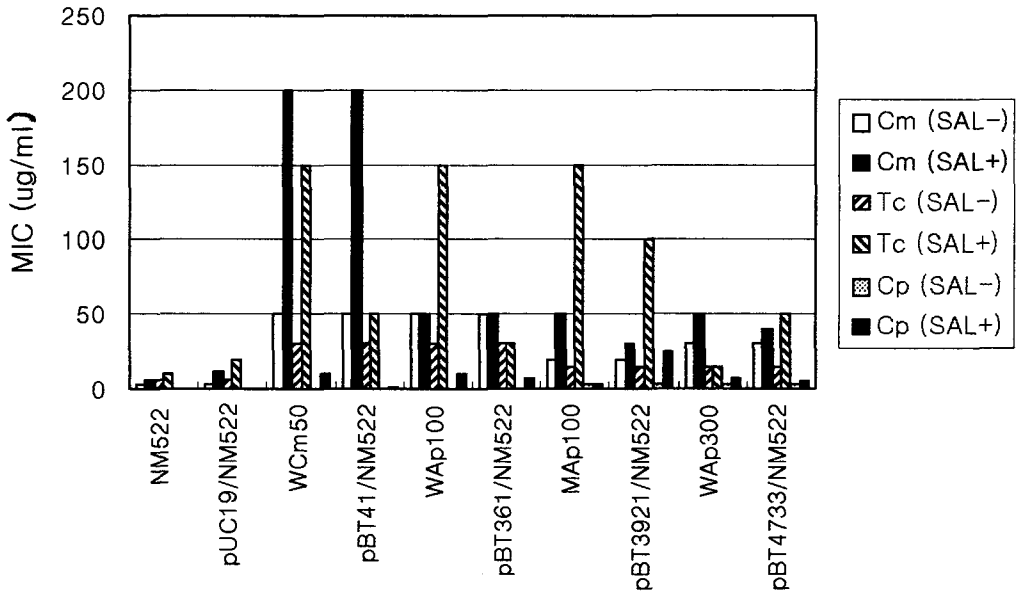


**Fig. 2.** Antimicrobial resistance of transduced *marRAB* mutation by λ*plac*Mu9 from *E. coli* Mar mutants (WCm50, WAp100, MAp100, or WAp300) into pUC19 cloning site (pBT41, pBT361, pBT3921, or pBT4733), respectively. pUC19/JM109 denotes the pUC19 plasmid in NM522 host strain. Ampicillin (Ap) resistant phenotype of each Mar mutants was not tested because of pUC19 ($Ap^r$ MIC = >900 ug/ml). Cm, chloramphenicol; Tc, tetracycline; Cp, ciprofloxacin. SAL+ means the culture with addition of salicylate (SAL); SAL-, without addition of SAL.

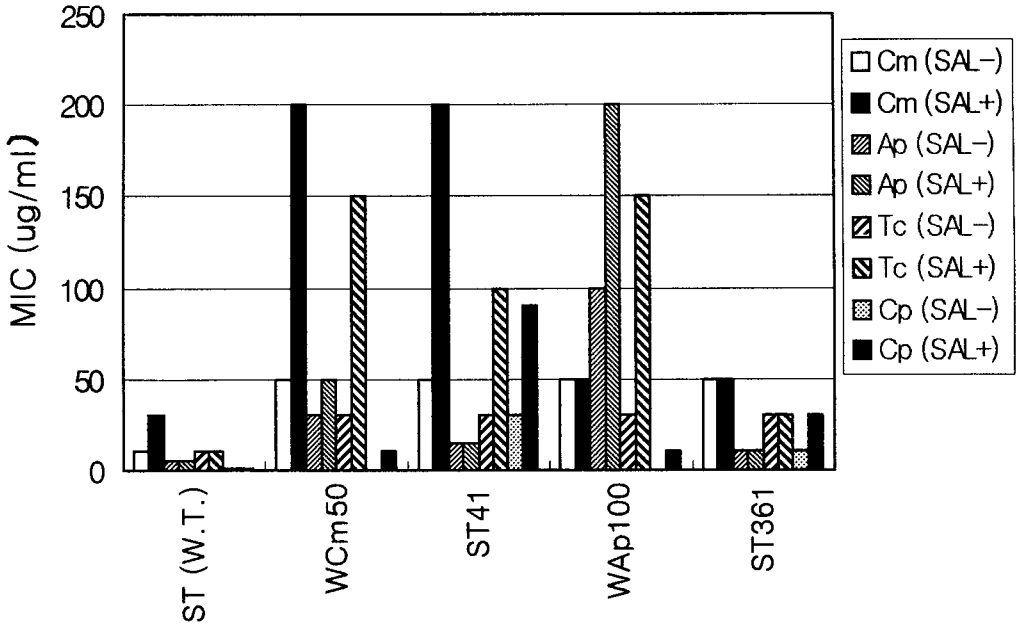


**Fig. 3.** Antimicrobial resistance of transduced *marRAB* mutation by λ*plac*Mu9 from *E. coli* Mar mutants (WCm50, WAp100) into choromosome of *S. typhimurium* (ST) TT10502 (ST41, ST361), respectively. W.T., wild type; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline; Cp, ciprofloxacin. SAL+ means the culture with addition of salicylate (SAL); SAL-, without addition of SAL.

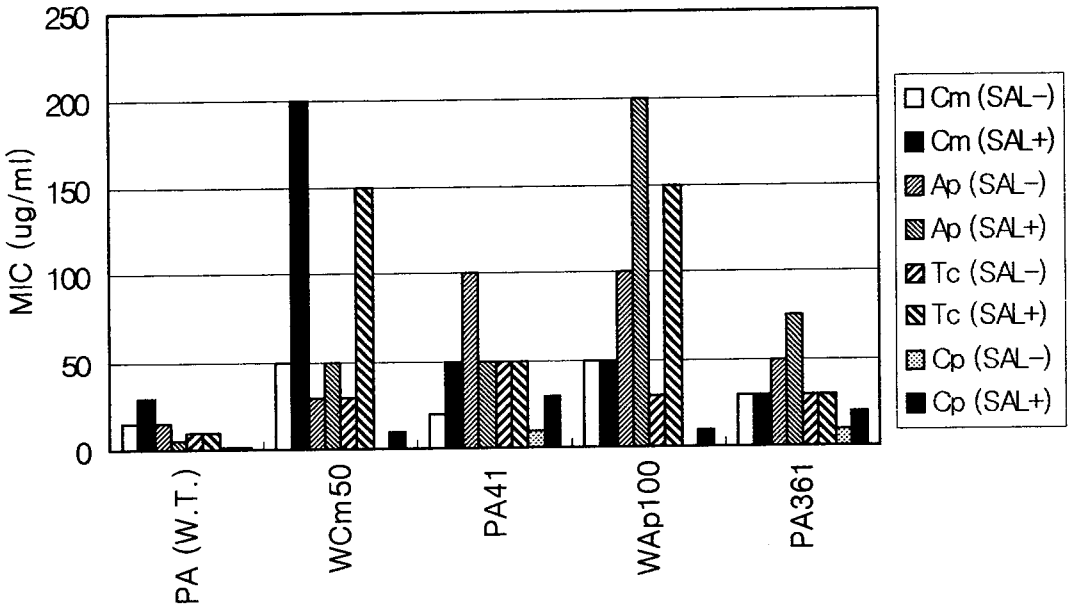


**Fig. 4.** Antimicrobial resistance of transduced *marRAB* mutation by λ*plac*Mu9 from *E. coli* Mar mutants (WCm50, WAp100) into choromosome of *P. aeruginosa* (PA) ATCC 27853 (PA41, PA361), respectively. W. T., wild type; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline; Cp, ciprofloxacin. SAL+ means the culture with addition of salicylate (SAL); SAL-, without addition of SAL.

Cp (Fig. 2) antibiotics, respectively.

**Transducion of *marRAB* mutation and SAL induction in another species of gram-negative bacilli: *Salmonella typhimurium* or *Pseudomonas aeruginosa***

To evaluate the spreading possibilities of the *marRAB* mutation of *E. coli* Mar mutant among another species of gram-negative bacilli, *marRAB* operon of *E. coli* Mar mutants (WCm 50 or WAp100) were transduced by λ*plac*Mu9 from the chromosome of Mar mutants into the wild type chromosome of *Salmonella typhimurium* TT10502 (Fig. 3) or *Pseudomonas aeruginosa* ATCC 27853 (Fig. 4). Compared with wild type *S. typhimirium* TT10502 or *P. aeruginosa* ATCC 27853, all transductants of ST 41, ST361, PA41, and PA361 exhibited higher levels of antimicrobial resistance and SAL induction of Mar phenotype. In contrast to the original Mar mutants of *E. coli*, there were some tendencies of increased antimicrobial resistance of all transductants with SAL induction against Cp (Fig. 3 and Fig. 4). However, decreased antimicrobial resistance with SAL induction against Ap and Tc (Fig. 3), or Cm and Tc (Fig. 4) antibiotics was shown, respectively.

## DISCUSSION

While many gram-negative bacteria have acquired their antimicrobial resistances on extrachromosomal elements, such as R plasmids or transposons, a number of clinical isolates show multiple-antibiotic-resistance (Mar) specified by the chromosome[8,15,36]. The chromosomal *mar* locus is located at 34 min on the *E. coli* genetic map, consists of *marO, marR, marA*, and *marB* genes, is induced by SAL. The *marRAB* operon of *Escherichia coli* is involved in the regulation of intrinsic resistance to many antimicrobials, structurally related or not, or toxic materials. Mutation of *marR* exhibits higher levels of antimicrobial resistance than wild type parents, and higher levels of antimicrobial resistance when induced by SAL than without induction by SAL[2,33].

In this study, transduced *marRAB* mutation into pUC19 cloning site in another strains (JM 109 or NM522) of *E. coli*, or onto the chromosome of *S. typhimurium* or *P. aeruginosa*, were evaluated for the spreading possibilities of *marRAB* mutation by antimicrobial susceptibility test, with or without addition of SAL. In case of *Providencia stuartii*[16], introduction of *aarP* (aminoglycoside acetyltransferase regultor) on a multicopy plasmid into either *P. stuartii* or *E. coli* conferred a Mar phenotype with higher levels of resistance to Cm, Tc, and ciprofloxacin (Cp). In this study, transduced *marRAB* mutation into pUC19 multicopy plasmid in another strains of wild type *E. coli*, JM109 or NM522, also showed a Mar phenotype with higher levels of resistance to Cm, Tc, and Cp than those of wild type strains. However, SAL inducibilities of JM109 or NM522 harboring pUC19::*marRAB* mutation against tested antibiotics were same or lower than those of original *E. coli* Mar mutants. According to the reports of Cohen et al.[8], Lewis et al.[15], Sulavik et al.[35], Zhanel et al.[36], *mar*-like DNA sequences or Mar phenotypes and their similar functions were located or expressed on or by the chromosome of a wide variety of bacterial species, such as, *E. coli, Salmonella* species, *Shigella* species, *P. aeruginosa, Hemophilus influenzae, Klebsiella* species, *Mycobacteria*, gonococcus, etc. This study also shows nearly the same results described above reports. All of the transduced *marRAB* mutation from *E. coli* Mar mutants onto the chromosome of *S. typhimurium* TT10502 or *P. aeruginosa* ATCC 27853 showed Mar phenotypes with higher levels of resistance to Cm, Ap, Tc, and Cp than those of wild type parents. However, SAL inducibilities of all transductants against tested antibiotics were same or lower than those of original Mar mutants of *E. coli*, except Cp, against which increased antimicrobial resistance with SAL induction was shown.

Important intercellular gene transfer methods between bacteria are transformation, transduction, or conjugation[3]. This study employed the method of transformation or transduction. Therefore, this study shows the evidences that suggest indirectly the spreading possibilities of *marRAB* mutation among gram-negative bacilli.

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=국문초록=

# Mar (Multiple-Antibiotic-Resistance) Operon 돌연변이 대장균의 그람음성 세균들간 전파 가능성에 대한 근거

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Mar (multiple-antibiotic-resistance) 대장균의 *marRAB* 돌연변이가 그람음성 세균들 사이로 전파될 수 있는지를 알아보기 위해서, 염색체 위의 *marRAB* 돌연변이를 λ*plac*Mu9 ($Km^r$)를 이용하여 대장균의 다른 균주내의 pUC19 ($Lac^+$, $Ap^r$)의 클로닝 사이트, *S. typhimurium* 및 *P. aeruginosa*의 염색체 위로 각각 전달시킨 후, Mar phenotype, $Km^r$, $Lac^-$, 혹은 $Ap^r$ 균주를 선택한 다음, salicylate (SAL)의 유도 (induction) 유무에 따른 항생제 내성검사를 실시하였다. pUC19의 보유 유무에 관계없이 대조군인 야생형 대장균 JM109, NM522에 비해서, pUC19::*marRAB* 돌연변이를 가진 JM109 혹은 NM522의 대부분 균주들은 항생제 내성 수준들이 훨씬 더 높았으며, Mar 표현형의 유도도 SAL에 의해서 훨씬 더 높게 나타났다. 하지만 일부 소수의 항생제에 대해서는 예외적으로 내성 수준이 더 낮게 나타났는데, 야생형 JM109 균주에서는 chloramphenicol (Cm)과 tetracycline (Tc)이, pUC19::*marRAB* 돌연변이를 가진 NM522 균주에서는 Tc와 ciprofloxacin (Cp)에 대한 내성 수준이 각각 더 낮게 나타났다. *S. typhimurium* 및 *P. aeruginosa*에 대한 같은 실험의 결과도 야생형 대장균의 경우와 거의 대부분 일치하였다. 형질전환 (transformation)과 형질도입 (transduction)의 방법을 주로 사용한 이 실험의 결과는, *marRAB* 돌연변이가 그람음성 세균들 사이로 전파될 수 있음을 간접적으로 암시한다.

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