

# Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity

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## ABSTRACT

**A new gene *mvrC* conferring resistance to methyl viologen, a powerful superoxide radical propagator, was cloned on 13.5 kilo base (kb) *EcoRI* DNA fragment. It gave resistance against methyl viologen to even a wild-type strain with gene dosage dependence. From the physical maps obtained by restriction enzyme digestions, it was predicted to locate at 580 kbp (12.3 min) on the physical map of *E.coli*. This was confirmed by the Southern hybridization of lambda phages covering this region with *mvrC* probe. The DNA sequence of *mvrC* gene was determined and its deduced protein encoding a 12 kd hydrophobic protein was confirmed by maxicell labeling of MvrC protein.**

## INTRODUCTION

Active oxygen species including superoxide anion radical, hydrogen peroxide, and hydroxyl radical, are continually produced during respiration. They are highly reactive and inactivate biological molecules such as DNA, proteins and lipids. They are, therefore, toxic to respiring cells. *Escherichia coli* has multiple defense mechanisms against active oxygen toxicity such as elimination of active oxygen species (1–6) and repair of damaged DNA by activated oxygens (7–14). And lately new repair mechanisms were proved to be induced after treatment with hydrogen peroxide and superoxide radical propagators such as methyl viologen (MV) and plumbagin (15–20). Virtually many proteins were induced following hydrogen peroxide and MV treatment (18,19,21–24) and some of their expressions were identified to be controlled by *oxyR* and *soxRS* regulator genes (18,19,21,22,25–27).

To elucidate the defense mechanisms against active oxygen toxicity, *E.coli* mutants sensitive to MV were isolated (28). Among them, new genes, *mvrA* and *mvrB*, were localized at 7 min and 27 min, respectively, on the linkage map of *E.coli*. They were highly sensitive to MV only in the presence of oxygen and the MV sensitivity was rescued by an addition of antioxidant uric acid, suggesting that the causal species of MV is superoxide radical. They were revealed to be involved in the same pathway of defense mechanisms against MV toxicity, because a double mutant strain, *mvrA mvrB*, was as sensitive to MV as a *mvrA*

strain. The *mvrA* gene, which could completely complement the sensitivity to MV of MC47 (*mvrA*) strain, was cloned and analyzed before (28). In this communication, a new gene *mvrC* was cloned and mapped at 12 min. It rescued the killing by MV of a *mvrA* mutant with gene dosage effect but not that by plumbagin. It was, therefore, designated *mvrC* gene. The DNA sequence was determined and its deduced amino acid sequence revealed that the MvrC protein was a hydrophobic protein. According to the program devised by Kyte and Doolittle (29), the MvrC protein can be classified into a group of membrane proteins. These results suggest that the *mvrC* gene, which codes for a membrane protein, exhibits resistance to MV by reducing permeability of MV. Another explanation is also discussed that the overexpression of the MvrC protein induces or activates the MvrA protein, which reduces the toxic species produced by MV.

## MATERIALS AND METHODS

### Bacterial strains and bacteriophages

Isolation and characterization of methyl viologen (MV)-sensitive strain MC47 was described before (28). It is shortly described as follows. Strain AB1157 was mutagenized with ethylmethane sulfonate and MV-sensitive mutants were isolated. Among them, a new mutant MMIX1-2 (*mvrA2*) was obtained and it was transduced into *E.coli* K12 strain AB2277 (*thi-1, ilvD145105 metE46, his-4, trp-3, proA2, mtl-1, malA1, ara-9, galK2, lacY1* or *lacZA, strA8* or *strA9, ton-1, tsx-3, supE44*), which was obtained from B.J.Bachmann (CGSC2277, Coli Genetic Stock Center, Yale Univ.). The new gene *mvrA* was identified to map at 7 min on the *E.coli* linkage map. MV was toxic only in the presence of oxygen and its toxicity was suppressed by the radical scavenger uric acid. Strain MC47 was only sensitive to MV and had a normal capacity for the MV-damaged DNA. From these results, strain MC47 was assumed to be impaired in the elimination of MV-specific toxic species.

Strain MG1063 was used for the analysis of *mvrC* gene size by integration of transposon Tn1000 into *mvrC* gene (30). Strain K280 carrying *uvrA48* and *recA1* mutations was constructed by mating Hfr *recA1* with AB2417 (*uvrA48*), which was supplied from P.Howard-Flanders. It was used for the identification of MvrC protein labeled by maxicell method (31). Bacteriophages

M13mp18 and M13mp19 were used for DNA sequencing (32). Lambda bacteriophages 2C4 ( $\lambda$ 158) and 8F11 ( $\lambda$ 159) carrying 12–13 min region of *E. coli* linkage map (33) were supplied from Y. Kohara (National Institute of Genetics, Mishima, Japan).

### Plasmids

Plasmid vectors used were pMF3, pBR322, pKK223-3 and pMC1871. The latter two were purchased from Pharmacia-Japan (Tokyo, Japan). Plasmid pMF3 is a stringent type and a low copy number vector derived from F factor (34). Plasmid pFMV2 is a derivative of pMF3 which carries 3.3 kbp *mvrC* containing DNA fragment derived from pMV2-1. Plasmid pKMV13 carrying only the ORF2 in Fig.6 was constructed with inserting 553 bp DNA fragment (450–1002 bp locus of the *mvrC* containing DNA fragment in Fig.6) into the *Sma*I site of pKK223-3 vector. It deleted the GTGAGT sequence of the original –35 region of the *mvrC* gene. But instead, at 17 bp upstream of the –10 region, it got the TTGGCT sequence as a new consensus sequence for the –35 region. Plasmid pMVC12 bearing only the ORF2 in Fig.6 was constructed with inserting 578 bp fragment (1–578 bp locus of the *mvrC* containing DNA in Fig.6) into the *Sma*I site of pMC1871.

### Chemicals and enzymes

Methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride, or paraquat) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Cloning enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Wako Chemicals (Tokyo, Japan) and were used according to the directions of the manufacturers. Radioisotopes of [ $^{32}$ P]dNTP mixture, [ $^{32}$ P]dCTP and [ $^{35}$ S]methionine were obtained from ICN Radiochemicals (Irvine, Calif.).

### Media

L broth contained 10g Bacto tryptone, 5g yeast extract and 5g NaCl per l. Agar media were solidified with 15g agar per l. MV medium contained methyl viologen (MV) at the indicated concentration in L broth. For the labeling of protein, M9 medium was used (34).

### Cloning of *mvrC* gene and its mapping

An *E. coli* gene bank was prepared by digestion of plasmid pBR322 and of *E. coli* W3110 DNA with the restriction enzyme *Eco*RI, followed by ligation. The MC47 transformants were selected by screening 0.2 mM MV- and 50  $\mu$ g tetracycline-resistant strains. Thus, plasmid pMV4 was obtained. It carried 13.5 kbp *Eco*RI DNA fragment which was localized from 574 to 588 kbp region on the *E. coli* physical map (33). Its derivatives, from pMV4-1 to pMV4-6, were constructed by partial digestion of pMV4 with *Pst*I, followed by self-ligation. The physical map of plasmid pMV4 conferring MV-resistance to strain MC47 (*mvrA*) was obtained by digestion with restriction enzymes *Bam*HI, *Hind*III, *Bgl*II, *Kpn*I, *Pst*I, and *Pvu*II. The localization of *mvrC* gene on the *E. coli* linkage map was predicted by comparing its physical map with the physical map of *E. coli* (33) and was determined by the Southern hybridization of lambda phage DNA covering this region with *mvrC* gene as a probe.

### Insertion of *Tn1000* into *mvrC* plasmid

This was done essentially the same methods as those of Guyer (30). A strain MG1063, which was transformed with plasmid pMV2-1 carrying *mvrC* gene, was mated with recipient strain MC47 *recA*I. Those recipient strains which became resistant to

both tetracycline and streptomycin were isolated. The transposon insertion points were determined from the restriction maps of *Tn1000* available (30) and of the *mvrC* gene that was examined.

### Analysis of plasmid-encoded proteins

Maxicells of strain K280(*recA48 uvrA1*) carrying plasmids pBR322, pMV2-1, and pMV2-1::*Tn1000* were prepared after UV irradiation and treatment with D-cycloserine (31). Plasmid-encoded proteins were labeled with 1  $\mu$ Ci/ml of [ $^{35}$ S]methionine by culturing maxicells in M9 medium supplemented with 30  $\mu$ g/ml of threonine, proline, and leucine. Then, they were analyzed by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE).

## RESULTS

### Cloning of *mvrC* gene and its property

During the cloning of *mvrA* gene to complement the MV-sensitivity of a *mvrA* strain, two kinds of plasmids, pMV1-1 and pMV2-1, were obtained (28). Plasmid pMV1-1 was identified to carry the *mvrA* gene (28). The plasmid pMV2-1 carrying *mvrC* gene could also rescue the killing of *mvrA* strain by MV toxicity and the suppression by pMV2-1 seemed to be more extent than that by plasmid pMV1-1 carrying *mvrA* gene. The suppression of MC47 strain by *mvrA* gene was gene dosage dependent; it was completely suppressed by a stringent type vector but partial by a relaxed type vector (28). Then, the gene dosage effect on the suppression of the sensitivity to MV was examined (Fig. 1). Plasmids pMF3 and its derivative pFMV2 carrying *mvrC* gene were a stringent type vector derived from F factor and there are 2–3 copies per cell. On the other hand, plasmid pMV2-1 is a

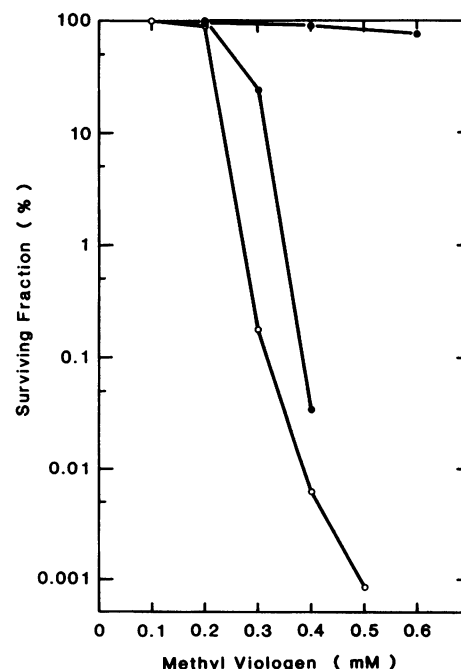


Fig. 1. The gene dosage effect of the *mvrC* gene on the suppression of the sensitivity to MV of strain MC47. Cells cultured to a stationary phase were spread on ampicillin-containing agar medium with or without MV at the indicated concentrations. After 2 or 3 days incubation, colonies were counted. MC47(pMF3), ○; MC47(pMV2-1), ⊙; ●.

derivative of pBR322 which is a relax type vector and there are 20 or more copies per cell. The suppression to MV was proportional to the copy number of the plasmids which carried the 3.3 kbp *Pst*I DNA fragment of plasmid pMV2-1, indicating

that the complementation by single *mvrC* gene is partial and indirect. The suppression of the sensitivity to redox-cycling drugs of strain MC48 was examined (Fig.2). The suppression to MV was also proportional to the copy number of the plasmids even

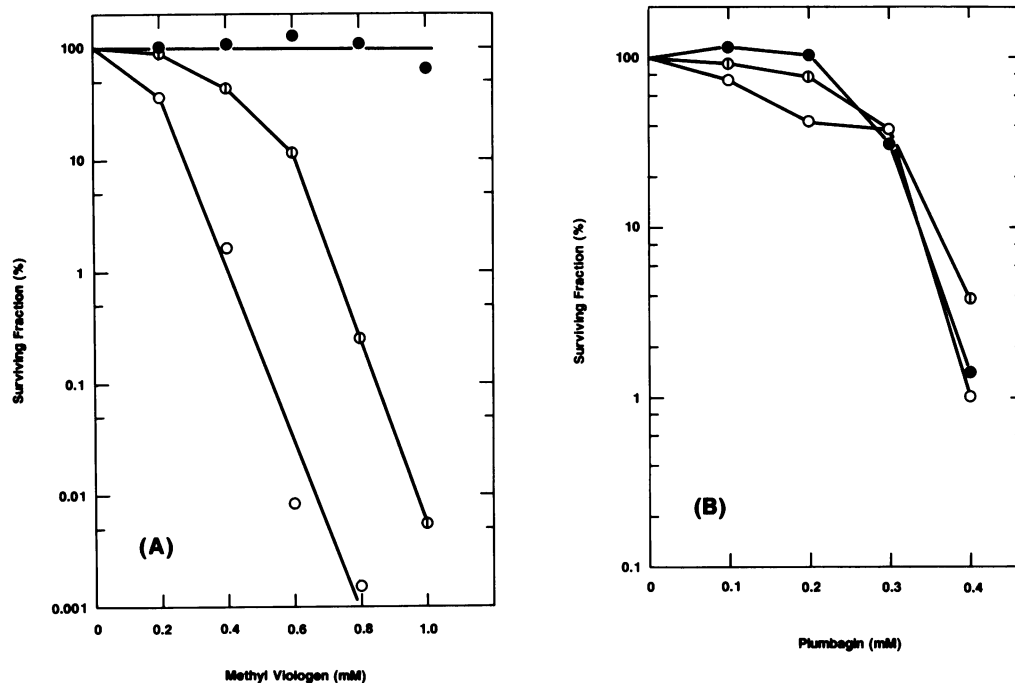


Fig.2. The *mvrC* gene effect on the resistance to MV (A) and to plumbagin (B) of a wild-type strain MC48. Methods were almost the same as described in the legend to Fig.1. MC48(pBR322), ○; MC48(pFMV2), ⊙; MC48(pMV2-1), ●.

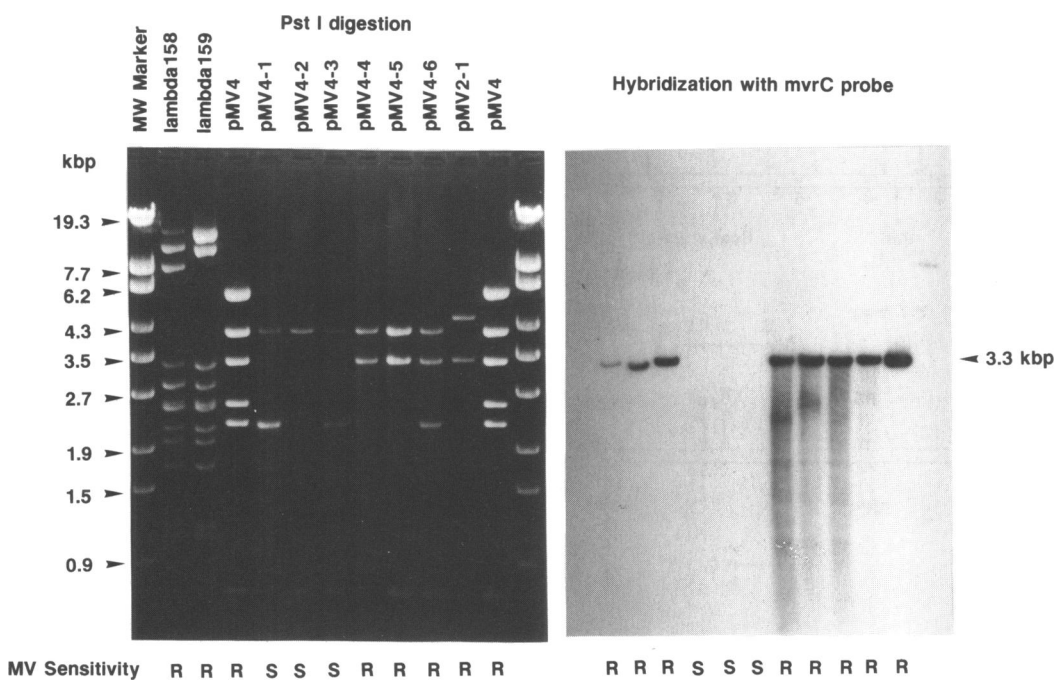


Fig.3. Southern hybridization. (a) Plasmid DNA digested with restriction enzyme *Pst*I were electrophoresed through agarose gel and photographed. (b) DNA blotted on a membrane filter was hybridized with *mvrC* probe obtained from pMV2-1 and autoradiography was done. R and S indicate resistance and sensitivity to MV, respectively.

when a wild-type strain was used, while the suppression to plumbagin which was also an active oxygen propagator, was the same even though the copy number changed from one to at least twenty. These results indicate that the 3.3 kbp *Pst*I DNA fragment carries the gene which specifically suppresses the sensitivity to MV of *E. coli*. The relevant gene was neither linked to *mvrA* at 7 min nor *mvrB* at 27 min by P1 transduction experiment (unpublished). That was, therefore, tentatively named *mvrC* gene.

**Mapping of *mvrC* gene**

Plasmid pMV2-1 carried 3.3 kbp *Pst*I DNA fragment, which had few sites for restriction enzymes used to construct the physical map of *E. coli* (33). *E. coli* had about 60 *Pst*I DNA fragments of the same 3.3 kbp size. This fragment is, therefore, too small to be localized on the physical map of *E. coli*. To know its locus on the *E. coli* linkage map, the longer DNA fragment containing

the *mvrC* gene was cloned. That was the plasmid pMV4, which is a derivative of plasmid pBR322 carrying 13.5 kbp *Eco*RI DNA fragment. It could complement the sensitivity to MV of a *mvrA* strain as well as plasmid pMV2-1. It also included the 3.3 kbp *Pst*I DNA fragment of the same size as plasmid pMV2-1. These results suggest that both plasmids have the same *Pst*I DNA fragment which can complement the MV sensitivity of a *mvrA* strain. The physical map of plasmid pMV4, which was determined by digestion with various restriction enzymes used in the construction of *E. coli* physical map (33), was completely the same as around 580 kbp locus on the *E. coli* physical map except the restriction sites by *Pvu*II. These results suggest that the *mvrC* gene is localized around 12.3 min on the *E. coli* linkage map. To confirm them, the Southern hybridization experiment was done (Fig.3). The *mvrC* probe obtained from plasmid pMV2-1 could hybridize with 3.3 kbp *Pst*I DNA fragment of plasmid pMV4 and its derivatives pMV4-4, pMV4-5 and pMV4-6, which were obtained by partial digestion with *Pst*I followed by self-ligation. Moreover, it also could hybridize with 3.3 kbp *Pst*I DNA fragments of  $\lambda$ 158 and  $\lambda$ 159, which were  $\lambda$  phages carrying 570–590 kbp region of *E. coli* physical map (33). This region was 12.3 min on the *E. coli* linkage map and was completely different from the sites of *mvrA* (7 min) and *mvrB* (27 min) genes (28). From these results the *mvrC* gene was identified to be a new gene which could specifically suppress the sensitivity to MV.

Plasmid	Restriction Map by <i>Dra</i> I	MV Sensitivity
pMV2-1		R
pMV21002		S
pMV21003		R
pMV21004		R
pMV21005		S
pMV21015		S
pMV21016		S
pMV21018		R
pMV21028		R

Fig.4. Deletion mapping of the *mvrC* gene. Plasmid pMV2-1 was partially digested with restriction enzyme *Dra*I and self-ligated. Open box represents the vector DNA of pBR322 and the closed one the cloned *E. coli* DNA. Arrows indicate the cutting sites by restriction enzyme *Dra*I.

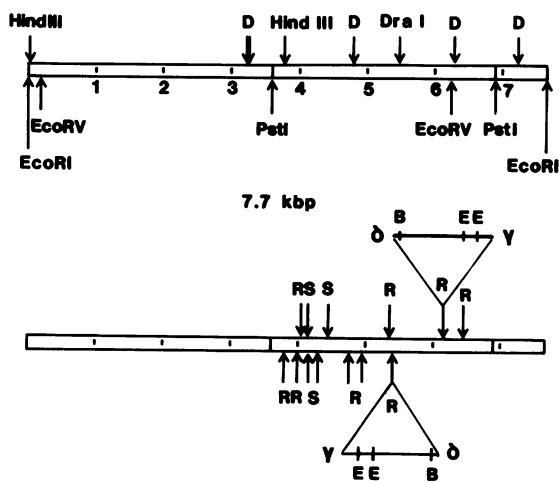


Fig.5. The localization of the *mvrC* gene on the physical map of plasmid pMV2-1 by transposon Tn1000 analysis. The integration sites of transposon Tn1000 determined were indicated as arrows. The transposons Tn1000 with  $\delta$   $\gamma$  direction were placed above the physical map and those with reverse direction  $\gamma$   $\delta$  were shown below it. Transposons Tn1000 of 5,700 bp are drawn not to scale. Restriction enzyme sites: B, *Bam*HI; D, *Dra*I; E, *Eco*RI;

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AAGCTTCTTTTCATTGCGTGAAGTGGAAATGTATCCATGGAGAACACTTTATCAGCCGGGA    60
AATAATGCGGGCAACGGTGTTTAATTATATATCGAATGTGATTACAATCGGTGCCGGCGGCA    120
CAGTTGTGTGGCGGCTCAGTCCGGAACAATTTGAAACAAGAACCCTCGCTTAGGCCCTG    180
TGTCATATTACGTGGGTAGGATCAACCAGCATAAATCAGGCTCGCGCTAAATGGCCGGC    240
fMetAlaArg
GGCAGGAATCCTAGTCGTGTGATGGTAAAGTCTGGCGAACGGTGTATTACCGGTTTCGCTAC    300
AlaGlyIleLeuValValAspGlyLysValTrpArgThrValTyrTyrArgPheAlaThr
CAGAGAAGAATGGGAAGAAAGGTGAGCACAATCTGATTTTAAAGGAGTGTCCAGAG    360
ArgGluGluTrpGluGlyLysValSerThrAsnLeuIlePheLysGluCysArgGlnSer
TGCCCGCATGAAACGGGTATTGAGGGTATATAAAGAACAATCAATGGGAACACAATGATG    420
AlaAlaMetLysArgValLeuArgValTyrLysArgThrSerMetGlyThrGln*op
AAACAGGTGAGTGTGAGTTCAAACTGTAGTACAATTCCTCCAGTTTGAACAGGAAGAAT    480
ATGCTATGAACCTTATATTATCTTGGTGGTCAACTTTCAGAGGTCATTGGTACAA    540
fMetAsnProTyrIleTyrLeuGlyGlyAlaIleLeuAlaGluValIleGlyThrThr
CCTTAATGAAGTTTTCAGAAGTTTTCACAGGTTATGGCCATCTGTGGTACAATTATT    600
LeuMetLysPheSerGluGlyPheThrArgLeuTrpProSerValGlyThrIleIleCys
GTTATGTGCATCTCTGGTATTAGCTCAGACCGTGGCTTATATTCCTACAGGGATTG    660
TyrCysAlaSerPheTrpLeuLeuAlaGlnThrLeuAlaTyrIleProThrGlyIleAla
CTTATGCTATCTGCTCAGGAGTCGGTATTGCTCTGATTAGCTTACTGTCATGGGATTTT    720
TyrAlaIleTrpSerGlyValGlyIleValLeuIleSerLeuLeuSerTrpGlyPhePhe
TCGCCCAACCGCTGGACCTGCCAGCATTATAGCCATGATGTGATTGTGCCGGTGTG    780
GlyGlnArgLeuAspLeuProAlaIleIleGlyMetMetLeuIleCysAlaGlyValLeu
TGATTATAATTATTATGTCACGAAGCACACCACATTAAATAATTTGTTCTAAACGACT    840
IleIleAsnLeuLeuSerArgSerThrProHis*oc
AAAATATGGAGGCTCTTATATTATATGAGCCTCGTTTATGCTTTTTGTTAATGCTTTT    900
ATTTTTATGATATTCTTTTGTGCTTCAAGATTATGGCGTAAGAAAATGCAATACGATT    960
ATTGTTGATATTCAGATAATGTGACCTAATTGTCTTTTT    1002
    
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Fig.6. The nucleotide sequence of the *mvrC* gene containing DNA fragment. The DNA sequence of the 1 kb *Hind*III-*Dra*I DNA fragment was determined by Sanger and Maxam-Gilbert methods. Open reading frames 1 and 2 are underlined in order with the deduced amino acid sequences. The consensus sequences of -10 and -35 regions are also underlined. Inverted repeats are indicated by a pair of arrows. The sequence 1–204 bp is completely the same as 1,054–1,258 bp of IS3 (39) and the sequence 205–391 bp is 90% homologous with that of lambda phage sequence 40,369–40,555 (40). The GenBank accession number is M62732.

**The physical structure of the *mvrC* gene**

The *mvrC* gene was more finely localized on the 3.3 kbp *Pst*I DNA fragment by deletion mapping and transposon Tn1000 integration analysis. The 3.3 kbp *Pst*I DNA fragment had three

cutting sites for restriction enzyme *Dra*I. Various deletion plasmids were obtained after partial digestion with *Dra*I and self-ligation of plasmid pMV2-1. The relationship between the loss of MV resistance and the loss of *Dra*I DNA fragment was examined (Fig.4). The loss of B fragment resulted in the loss of MV resistance and the presence of only B fragment was enough to exhibit the MV resistance, indicating that the *mvrC* gene is present in B fragment of *Dra*I digests of plasmid pMV2-1. Then, the *mvrC* locus was more finely mapped by transposon Tn1000 integration analysis (Fig.5). The insertion points of transposon Tn1000 were determined by analyzing the restriction fragments of plasmids pMV21::Tn1000 digested with *Eco*RI and *Bam*HI, as described by Guyer (30). It was also confirmed that the *mvrC* locus was in B fragment of *Dra*I digests of pMV2-1. The maximum range of the *mvrC* gene inactivated by their insertions was 300 bp, and the minimum range of the *mvrC* gene not inactivated was 600 bp. It is, therefore, concluded that the size of the *mvrC* gene is between 300 and 600 bp which is localized in the B fragment of *Dra*I digests.

**DNA sequencing of *mvrC* gene**

As the *mvrC* gene was localized within 1 kbp *Hind*III-*Dra*I DNA fragment (Fig.5), the DNA sequence of this fragment was determined by Sanger (32) and Maxam-Gilbert (36) methods. The DNA sequences obtained were completely the same and were shown in Fig.6. From the DNA sequence of 1,002 bp, there could be 13 open reading frames (ORF) on one strand and 8 ORFs on the complementary strand. Among them, either ORF1 of 186 bp plus ORF2 of 333 bp or single ORF2 was implicated to be *mvrC* gene, because they satisfied the *mvrC* gene size of 300–600 bp predicted from transposon Tn1000 analysis. Then plasmids carrying ORF1 and ORF2 were constructed and the suppression of sensitivity to MV was examined (Fig.7). Plasmid pKMV13 carrying single ORF2 rescued the killing of a *mvrA* strain by MV as well as plasmid pMV2-1, whereas plasmid pMVC12 carrying single ORF1 could not do so, indicating that ORF2 is

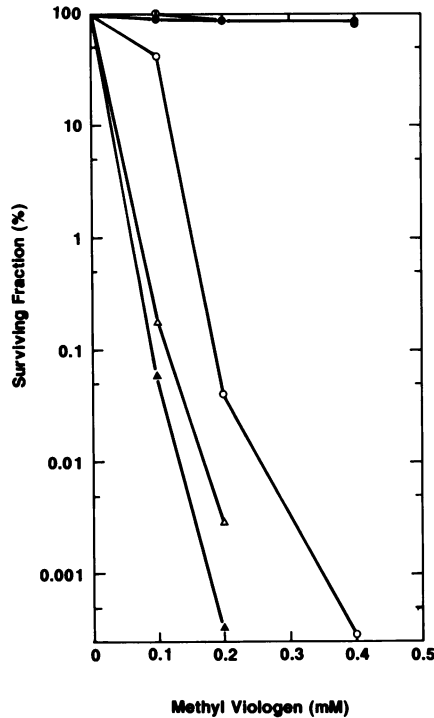


Fig.7. Survival curves of strain MC47 carrying ORF1 or ORF2 plasmid after plated on LB agar medium containing MV. MC47(pK223-3), ○; MC47(pK13) carrying ORF2, ⊕; MC47(pMV2-1) carrying ORF1 and ORF2, ●; MC47(pMC1871), △; MC47(pMVC12) carrying ORF1, ▲.

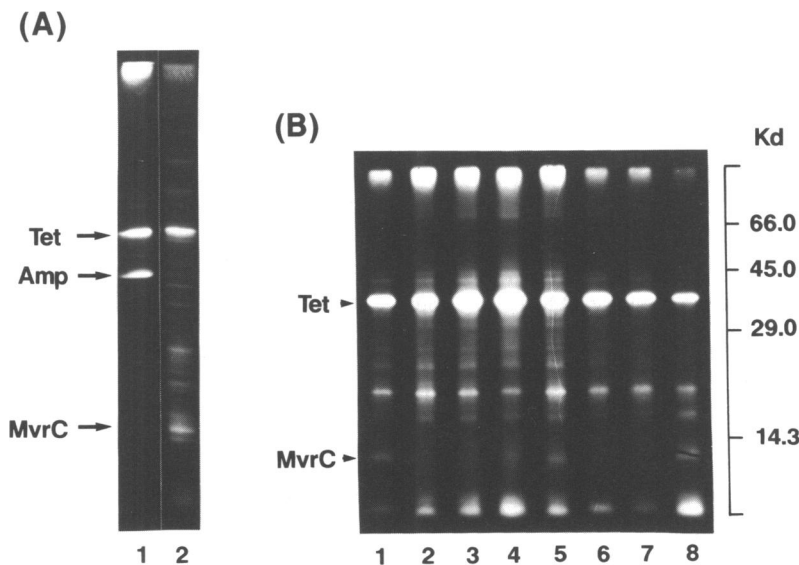


Fig.8. SDS-PAGE of plasmid-encoded proteins. Plasmid-encoded proteins were labeled with [<sup>35</sup>S]methionine after maxicell preparation and were analyzed by SDS-PAGE. (A) 1, pBR322, MV<sup>s</sup>; 2, pMV2-1, MV<sup>r</sup>. (B) Plasmids pMV2-1::Tn1000. 1, pMV2101, MV<sup>r</sup>; 2, pMV2103, MV<sup>s</sup>; 3, pMV2106, MV<sup>s</sup>; 4, pMV2107, MV<sup>s</sup>; 5, pMV2109, MV<sup>r</sup>; 6, pMV2110, MV<sup>s</sup>; 7, pMV2111, MV<sup>s</sup>; 8, pMV2121, MV<sup>r</sup>. MV<sup>r</sup> and MV<sup>s</sup> indicate the resistance and sensitivity to MV, respectively.

the *mvrC* gene. The ORF2 is 333 bp and encodes a 12,011 dalton protein with the promoter consensus sequences of GTGAGT, TA-CAAT and a ribosome-binding site of AGGA. Moreover, downstream this coding region there is a T-rich loop with a GC stem, which is a consensus sequence for a rho-independent transcription terminator. These results indicate that the ORF2 has a typical gene structure to be translated into a protein.

### MvrC protein

From the DNA sequence of *mvrC* gene, the size of deduced MvrC protein is predicted to be 12 kilo dalton (kd). The MvrC protein was identified by fluorography after it was labeled with [<sup>35</sup>S]methionine in maxicells and electrophoresed through SDS-PAGE (Fig. 8). Plasmid pBR322 produced Amp and Tet proteins, while plasmid pMV2-1 newly produced 1.2 kd protein with losing Amp protein (Fig. 8A). That was because the *mvrC* gene was cloned at *Pst*I site in *amp* gene. Plasmids such as pMV2101, pMV2109, and pMV2121, carrying intact *mvrC* gene produced 1.2 kd protein, while plasmids pMV2103, pMV2106, pMV2107, pMV2110, and pMV2111, bearing defective *mvrC* gene inserted by transposon Tn1000 lost this band (Fig. 8B), indicating that the size of MvrC protein is 12 kd. This is in good agreement with the molecular weight of the MvrC protein deduced from DNA sequence.

To know the property of MvrC protein to suppress the sensitivity to MV, the hydrophobicity of MvrC protein was calculated according to the program devised by Kyte and Doolittle (29). The mean hydropathy of the MvrC protein was 1.04, indicating that the MvrC protein is hydrophobic and can be classified into a group of membrane proteins. These results suggest that the *mvrC* gene codes for a membrane protein of 12 kd which specifically prevents the incorporation of MV into cells, with suppressing the sensitivity of a *mvrA* strain to MV.

### DISCUSSION

The *mvrC* gene was identified to be a new gene which could suppress the sensitivity of a *mvrA* strain to MV toxicity. It was cloned and mapped at 12.3 min on the *E. coli* physical map (33). It was different from the locus of the *mvrA* gene mapped at 7 min (28). These results indicate that the sensitivity of a *mvrA* strain to MV toxicity is functionally relieved by the *mvrC* gene. The hydrophobicity of the MvrC protein calculated from its amino acid composition indicates that the *mvrC* gene codes for a membrane protein (29), suggesting that the sensitivity of a *mvrA* strain to MV toxicity is rescued by preventing the incorporation of MV into cells. This was supported from the evidence that the *mvrC* gene specifically suppressed the sensitivity of a *mvrA* strain to MV toxicity but not to plumbagin toxicity. Moreover, the degree of suppression was proportional to the gene dosage. Namely, even a wild-type strain became more resistant to MV when the *mvrC* gene was cloned on a high copy number plasmid. These results indicate that the *mvrC* gene encodes a membrane protein which is impermeable to MV and gives resistance against MV even to a wild-type strain.

Methyl viologen is a derivative of viologen, a positively charged biphenyl, which is thought to require energy to be incorporated into cells. Kao and Hassan (37) reported the isolation of MV-tolerant *E. coli* strain AI-11 which was reduced in the active transport of MV into cells, suggesting that it is impaired in a membrane protein involved in the permeability of MV. It could be a mutant with reverse phenotype of *mvrC* gene. It is,

therefore, likely that their MV-tolerant strain AI-11 is a constitutive mutant or overproduction mutant of *mvrC* gene. This possibility seems likely but it must wait to conclude it. The isolation of *mvrC* mutant strain and analysis of MV uptake will provide insight into this hypothesis.

Another explanation on the function of the MvrC protein is that the overexpression of the MvrC protein induces or activates the MvrA protein which encodes a soluble protein, probably an enzyme (28), and reduces the toxic species produced by MV. It is a very attractive hypothesis but the function of the MvrA protein must be solved first. As for the function of the MvrA protein, we personally received a letter from Peter Reichard at Karolinska Institute, Sweden, that a component of ribonucleotide reductase, which is induced under anaerobic conditions, was purified and had a very similar amino acid sequence to the sequence of the MvrA protein. When reducing ribonucleotide, it uses an organic free radical (38). The MvrA protein might play a role to enhance reduction of toxic species produced by MV. The overproduction of the MvrC protein could induce or activate the MvrA protein which rescue *E. coli* from killing by toxic species produced by MV.

The DNA sequence of 1,002 bp including the 333 bp of *mvrC* gene was determined. The homology search revealed that there was no homologous gene with the *mvrC* gene. On the contrary, there were IS3 and lambda homologous sequences in the flanking region of the *mvrC* gene. The DNA sequence of 1–204 bp locus in Fig. 6 was completely the same as the 1,054–1,258 bp locus of the IS3 DNA sequence (39), indicating that the insertion sequence IS3 is integrated around 580 kbp on the physical map of *E. coli*. The 204–391 bp region of the *mvrC* containing sequence in Fig. 6 was about 90% homologous with the 40,369–40,555 bp locus of lambda DNA sequence (40), suggesting the abortive excision of lambda phage integrated at this region before.

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