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Effects of R100 Mutant MerR on Regulation of *mer* Operon from *Shigella flexneri*

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An amino-terminal 14 amino acids deletion and three site-directed mutations were created to investigate the mechanism of induction and repression of MerR regulatory protein in R100 *mer* operon from gram-negative *Shigella flexneri*. The amino-terminal 14 amino acids deletion,Cys117Ser, and Cys126Ser mutations abolished the inducibility of the *mer* operon and the His118Ala mutation resulted in the reduction of inducibility (about 9.1% remaining) in complementation experiment in the presence of Hg^{2+} at subtoxic level (1 μM). The complementation experiment with Hg^{2+} absent showed that His118Ala, Cys126Ser, and wild-type MerR could repress the operon but Cys117Ser could not, and the amino-terminal deletion mutant could neither induce nor repress the R100 *mer* operon.

The mechanisms through which the bacteria turn genes on and off in response to metal availability has become the subject of intensive research in recent years. The bacterial mercury-resistance (*mer*) operon confers resistance to inorganic mercury (Hg^{2+}) (1, 3, 13, 16, 17, 19, 20). The most extensively studied example of *merR* is encoded by the gram-positive chromosomal *merR* from *Bacillus subtilis* RC607 (7, 13). The *mer* operon also exists in the R100 plasmid from a gram-negative *S. flexneri*. The *mer* consists of five structural genes and one regulatory gene: *merT, merP, merC, merA, merD,* and *merR*. Three genes (*merT, merP,* and *merC*) are involved in transport of mercury. The MerT and MerC polypeptides are inner membrane protein, whereas the MerP polypeptide is a periplasmic protein (3, 16, 17). One (*merA*, the mercuric reductase) gene is involved in the enzymatic reduction of toxic mercury (3, 16). A small, low-abundance protein (*merD*) whose function is now under investigation. The expressionof *merTPCAD* is under negative control in the absence of Hg^{2+} and under positive control in the presence of Hg^{2+} by the product of the *merR* gene(3, 4, 5, 9, 16). MerR also negatively regulates its own synthesis in the absence of Hg^{2+} (4, 7, 16). The proposed helix-turn-helix structure and His and Cys residues are evolutionally conserved in all sequenced MerR proteins. The helix-turn-helix structure seemed to be needed by the regulatory protein in order to bind itself to the operator, and the Cys and His are obvious candidates for Hg^{2+} ligands considering the strength of Hg-Cys and Hg-His bonds. For this reason, amino-terminal 14 amino acids were deleted to remove the proposed helix-turn-helix structure to examine the role of helix-turn-helix. Histidine 118, Cysteine 117, and Cysteine 126 were replaced with alanine, serine, and serine respectively by site-directed mutagenesis to exmine the function of histidine and cysteine residues of the R100 MerR protein in the inducibility and repression of *mer* operon.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids

Table 1 lists the bacterial strains, and plasmids used. All strains were stored frozen at -70°C. Two sets of frozen cultures were stored separately in two different deep freezers.

Growth Media and Growth Conditions

JM105 were grown on a M9 medium (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 50 μM $CaCl_2$) supplemented with 0.2% glucose and thiamine (10 μg/ml) at 37°C (15). All other *E. coli* strains were grown on a Luria Broth medium (15). The medium was supplemented with ampicillin(100 μg/ml), or kanamycin (50 μg/ml), or both as required.

Plasmid DNA Preparations

Plasmid DNA preparation, precise quantitation, modification of DNA fragment, and gel electrophoresis were performed by the method described by Sambrook et al. (15).

Table 1. Bacterial strains and plasmids

Strains or plasmids	Relevant genotype or description	Reference or source
E. coli strains		
JM105	△(*lac-proAB*) *thi strA rpsL endA sbcB15 hsdR4* (F'*traD36 proA*$^+$*B*$^+$ *lacI*^{q} Z△*M15*)	16
MK30-3(SupE$^-$)	△(*lac-proAB*) *recA galE strA* (F'*proA*$^+$*B*$^+$ *lacI*^{q} Z△*M15*)	11
Phages		
M13mp18, M13mp19	Templates for DNA sequencing and Mutagenesis	17
M13mp9am16	An amber mutation in codon 16 of M13mp9	11
TM001	An 620 bp *Eco*RI-*Hin*dIII R100 *merR* fragment in M13mp19	10
Plasmids		
pDU1161	Ka^r Cm^r Hg^s R100 *merR*::Tn*5 merA*::Mu d *amp lac*	18
R100	Hg^r plasmid from *S. flexneri*	10
pKK223-3	Amp^r Overexpression vector with *tac* promoter	Pharmacia LKB
pKPY1000	R100 *merR* (wild-type) in pKK223-3	This study
pKPY1001	R100 mutant (Cys117Ser) in pKK223-3	This study
pKPY1002	R100 mutant (Cys126Ser) in pKK223-3	This study
pKPY1003	R100 mutant (His118Ala) in pKK223-3	This study
pKPY1004	R100 mutant (N-terminal 14 amino acids missing) in pKK223-3	This study

Site-Directed Mutagenesis

A 620 bp *Eco*RI-*Hin*dIII fragment containing a wild-type *merR* was isolated from pTM001 (2) and cloned into the *Eco*RI and *Hin*dIII sites of M13mp9 amber bacteriophage to prepare ssDNA template for mutagenesis by the methods as described (8). A gapped duplex was formed by mixing 0.5 pmol of ssDNA, 0.1 pmol of *Eco*RI-*Hin*dIII linearized mp19 DNA in a total volume of 40 μl (0.2 M KCl, 0.015 M Tris, pH 7.5). The mixture was heated at 100°C for 3 min and 60°C for 5 min, then was slowly cooled to room temperature. To anneal primers to the gapped duplex DNA template, 16 μl of gapped duplex and 4 μl of kinased primers (2 pmol) were mixed and the temperature was brought up to 65°C for 5 min, and it was decreased slowly to room temperature. To 10 μl of gapped duplex and primer, 4 μl of 10X PEL buffer (1.0 M KCl, 0.3 M Tris, 0.15 M $MgCl_2$, 0.02 M DTT, pH7.5), 3 μl of dNTPs (0.25 mM solution made from 10 mM stocks), 3 μl of ATP (10 μl of 0.1 M ATP stock+1490 μl of H_2O), 17 μl of ddH_2O, 5 U of DNA polymerase I Klenow fragment, and 10 U of DNA ligase were mixed and incubated at 16°C for overnight. For transformation, 10 μl of extention/ligation mixture was added to 200 μl of freshly prepared competent *E. coli* MK30-3 (SupE$^-$) cells, and this was kept on ice for 30min, and at 42°C for 1 min for heat shock. Various amounts of transformation mixtures were mixed with 100 μl of fresh MK30-3 (SupE$^-$) and 3 ml of soft agar, and they were plated on minimal medium agar plates. The plates were incubated at 37°C for one or two days. To identify the mutant, 3 to 5 colonies were randomly selected and sequenced. The 620 bp *Eco*RI-*Hin*dIII fragment was isolated from the identified mutant and cloned into the *Eco*RI-*Hin*dIII sites of pKK223-3 overexpression vector subsequently.

Preparation of pKPY1004

Amino-terminal 14 amino acids of R100 (proposed helix-turn-helix site) *merR* were deleted in the following way. TM001 (Table 1) was linearized by *Eag*I whose site was present in *merR* only once (in 620 bp *Eco*RI-*Hin*dIII fragment), and the resulting 5' protruding ends were filled by DNA polymerase I Klenow fragment. After the ligation of synthetic *Cla*I linker ($^{5'CCCATCGATGGG3'}_{3'GGGTAGCTACCC5'}$) to the polished ends, *Cla*I digestion and religation by T4 DNA ligases were carried out. An *Eco*RI-*Hin*dIII fragment (about 640 bp) was isolated from the modified TM001 after electrophoresis in a 1% agarose gel, and cloned into the *Eco*RI-*Hin*dIII sites of pUC19. Then the resulting plasmid was cut by *Cla*I and blunt-ended by DNA polymerase I Klenow fragment. *Hin*dIII linker($^{5'CAAGCTTG3'}_{3'GTTCGAAC5'}$) was added at the polished ends. These combined DNA modifications generated on ATG sequence for the new NH_2 terminal (N-terminal methionine). A 650 bp *Eco*RI-*Hin*dIII fragment was isolated from it, sequenced to verify the exact sequence, and subsequently cloned into the *Eco*RI-*Hin*dIII sites of pKK223-3. The resulting recombinant plasmid was named pKPY1004.

β-galactosidase Assays

β-galactosidase assays were carried out as described (6). The cultures were grown overnight in 5 ml of LB containing suitable antibiotics. The overnight culture was diluted 100-fold in fresh LB and the diluted culture was incubated at 37°C for 2-3 h until OD_{600} reached 0.5. When inducing with mercuric salts to be studied, different concentrations of $HgCl_2$ or phenylmercury acetate (0.1, 0.2, 0.5, 1.0, or 2.0 μM [PMA]) were added 60 min before the β-galactosidase assay. Spectrophotometric reading at OD_{420}, OD_{550}, and OD_{600} were measured and

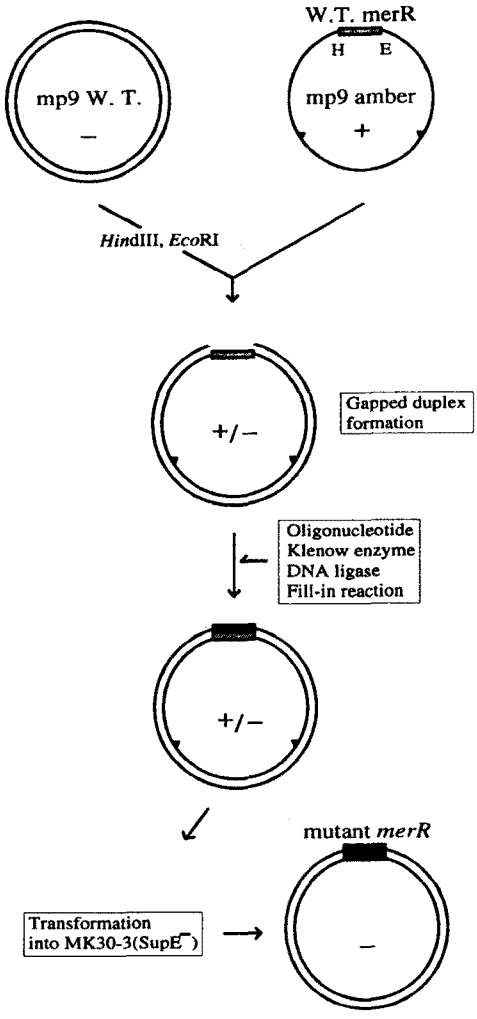

Fig. 1. Gapped-duplex method of site-directed mutagenesis. gpDNA is formed by annealing linearized double-stranded (RF) DNA to an excess of circular single-stranded recombinant DNA containing an insert [(+) strand]. After hybridization, the oligonucleotide is joined to the(-) strand by enzymatic filling-in and ligation. After transformation with the hybrid molecule, only the phage without the amber mutations [the (-) strand containing the site-specific mutation will be propagated in a suppresor-negative strain.

the β-galactosidase activity was calculated subsequently.

RESULTS AND DISCUSSION

Site-Directed Mutagenesis of R100 MerR

The "gapped duplex" method was used for site-directed mutagenesis as described (8). The desired mutations were created by hybridizing mutagenic oligonucleotide (Table 2) to the gapped duplex. After transformating the hybrid molecule into a suppressor-negative strain (MK30-3, SupE-), only the phage without the amber mutations (the [−] strand, which also contains the site-specific mutation) were propagated. All of the mutations were verified by sequencing and cloned into a pKK223-3 plasmid and named pKPY1001, pKPY1002, and pKPY1003, respectively.

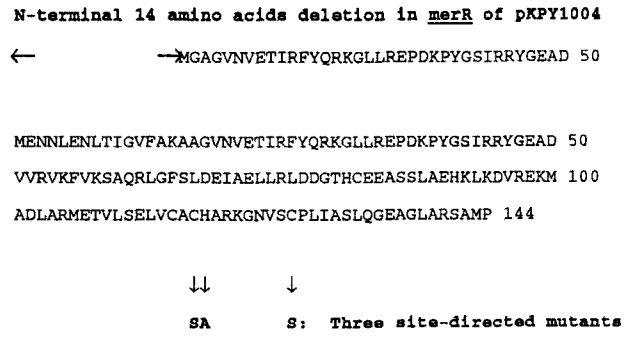

Fig. 2. Mutations in R100 *merR*.
An amino-terminal 14 amino acids deletion mutant and three site-directed mutants were shown.

Effects of Amino-Terminal Deletion, His118Ala, Cys 117Ser, and Cys126Ser of R100 MerR on Induction of R100 *mer* Operon by Mercuric Ion

Effects of various mutations on the induction of R100 *mer* operon were studied in complementation experiments between pDU1161 (R100 *merR*::Tn*5* *merA*::mu d *amp lac* Cm^r Km^r Hg^s) and wild-type *merR* and mutant *merR* (Fig. 3, 4; Table 2) in JM105. In this experiment, a *merA-lacZ* fusion protein was produced as the result of R100 *mer* operator-promoter activity on pDU1161, and the *mer* operator-promoter activity was induced by MerR from other plasmid (pKPY) in the presence of mercuric ion at subtoxic level and repressed by the same MerR in the absence of Mercuric ion (Fig. 3). Cells containing the appropriate plasmid (pDU1161 alone or pDU1161 and different type of *merR* for complementation) were grown and induced by 0. 0.5, 1.0, 1.5, 2.0 μM of $HgCl_2$. When pDU1161 was present in JM105 alone, the basal level of specific β-galactosidase activity was expressed constitutively about 195 miller units. When the wild-type R100 *merR* (pKPY1000) was present

Table 2. Oligomers used for site-directed mutagenesis

Mutant	Wild-Type	Mutation	Oligomer
Cys126Ser	Cys[a]	Ser[c]	5'TCAACGGGCTGGAAACA3'
Cys117Ser	Cys	Ser	5'GTGCATGGCTGGCGCAC3'
His118Ala	His[b]	Ala[d]	5'TTTCGTGCAGCGCAGGCGCA3'

[a] Cysteine, [b] Histidine, [c] Serine, [d] Alanine.

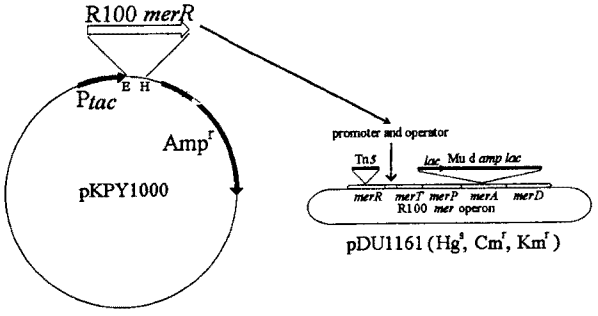

Fig. 3. Complementation experiment between pDU1161 and *merR*.
Plasmid pDU1161 has Tn5 insertion in R100 *merR* and mu d *amp lac* insertionin *merA* so that it can make MerA-Lac fusion protein upon induction. pKPY1000 has R100 *merR* cloned into the *Eco*R1-*Hin*dIII site of pKK223-3. Experiments were carried out in JM105.

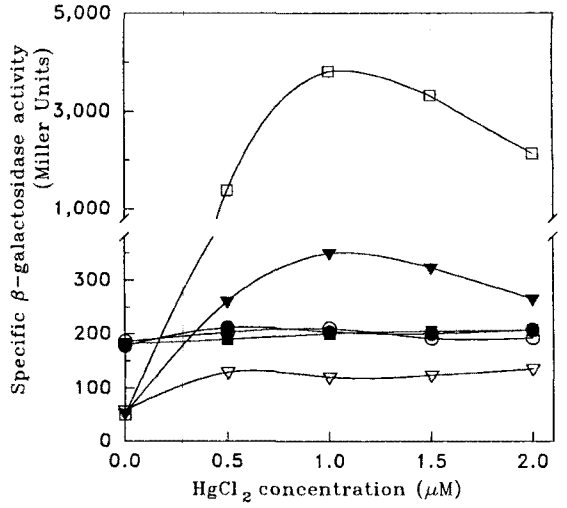

Fig. 4. Induction of *mer* operon by mercuric ion.
Early exponential JM105 harboring appropriate plasmid were induced by different concentrations of $HgCl_2$ (0.1, 0.2, 0.5, 1.0, 2.0 μM) at 37°C for 60 min. β-galactosidase activity were calculated as described (12). pDU1161 alone (○); pDU1161 and pKPY1000 (□); pDU1161 and pKPY1001(●); pDU1161 and pKPY1002 (▽); pDU1161 and pKPY1003 (▼); pDU1161and pKPY1004 (■).

together with pDU1161 in 1 μM of Hg^{2+}, the *mer* promoter of pDU1161 was activated by 77 folds and the specific β-galactosidase activity reached 3827 miller units (Fig. 4). But His118Ala could activate the promoter by only about 6.5 folds and the specific β-galactosidase activity reached only 315 units (about 9.1% remaining from 3827 units) in 1 μM of Hg^{2+}. Cys117Ser, Cys126Ser, and amino-terminal deletion mutant could not induce the R100 *mer* operon at all (Fig 4). At a higher concentration (1.5 μM and 2.0 μM) of mercuric ion, the specific β-galactosidase activity was slightly reduced due to the toxicity of mercuric ion. The failure of Cys117Ser and Cys126Ser to induce the *mer* operon suggested that these amino acids might be required for activation by mercuric ion. His-118 (which is located right next to Cys-117) was also important in induction of *mer* operon. Protein crystallography data would be necessary to explain any possible interactions between Hg^{2+} and these amino acids.

Effects of Amino-Terminal Deletion, His118Ala, Cys 117Ser, and Cys126Ser of R100 MerR on Induction of R100 *mer* Operon by PMA

An organomercurial like PMA was shown to induce mercuric and organomercurial resistance determinants of plasmid pDU1358 (6). Though R100 *mer* operon does not confer resistance to organomercurials due to the absence of *merB* which is an organomercuric lyase (6, 12), it would be interesting to know if there is any possibility of R100 *mer* operon being induced by organomercurial like PMA. Cells containing the appropriate plasmid (pDU1161 alone or pDU1161 and a different type of R100 *merR* for complementation) were grown and induced by 0. 0.5, 1.0, 1.5, 2.0 μM of PMA. In this experiment, the β-galactosidase activities of pDU1161 alone or pDU1161 with Cys117Ser reduced as the concentration of PMA increased due to the toxicity of PMA. The previously observed repression (Fig. 4) was repeated in the case of wild-type MerR and Cys126Ser. The concentration of PMA might not be high enough to diminish these basal small β-galactosidase activities of repressed *mer* in pDU1161 by either MerR or Cys126Ser. These results suggested that PMA can not activate MerR. A broad-spectrum *merR* of pDU1358 has been shown to have a quite different carboxyl-terminal amino acids sequence (14, Fig. 3 in the accompanying paper). The unique carboxyl-terminal sequence may be needed for the inducibility by organomercurials.

Effects of Amino-Terminal Deletion, His118Ala, Cys 117Ser, and Cys126Ser of MerR on R100 *mer* Operon Repression

Since the wild-type Tn*21* MerR protein exists as a repressor in the absence of Hg^{2+} (4, 14, 16, 17), complementation experiments were carried out with pDU1161 to examine the effect of various mutations on repression. Complementation experiment with pDU1161 in the absence of Hg^{2+} showed that pKPY1004 could neither induce nor repress the R100 *mer* operon (Fig. 4). The loss of amino-terminal 14 amino acids might greatly impair the folding of MerR protein in such a manner that it could neither bind to the operator sequence nor induce R100 *mer* operon. The experiment also showed that His118Ala, and wild-type MerR can repress the operon,but that Cys117Ser does not have any effect. These results strongly suggested Cys126Ser, His118Ala, and the wild-type could bind to the R100 operator sequence, resulting in the reduced level of β-galactosidase activity. But Cys117Ser failed to bind to the

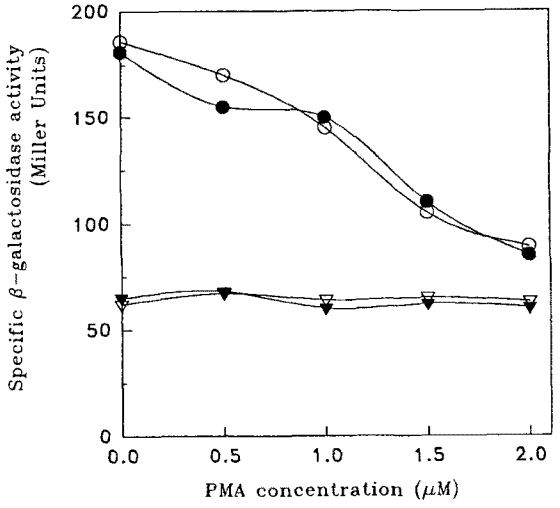

Fig. 5. Effects of phenylmercuric acetate on *mer* operon. Early exponential JM105 harboring appropriate plasmid were induced by different concentrations of PMA (0.1, 0.2, 0.5, 1.0, 2.0 μM) at 37°C for 60 min. β-galactosidase activity were calculated as described (12). pDU1161 alone (○); pDU1161 and pKPY1000 (▼); pDU1161 and pKPY100 (●);pDU1161 and pKPY1002 (▽).

operator region, resulting in the same level of β-galactosidase activity as that of pDU1161 alone. The site-directed mutation from Cys-117 to Ser might unstablize MerR protein, so that it can not form the dimer needed for binding of MerR to the operator sequence (4, 14, 16, 17). Another possible explanation is that Cys-117 might be required to form a disulfide bridge with other Cys in R100 MerR or that it might be involved in forming important hydrogen bonds with other amino acids of R100 MerR protein. A fine X-ray crystallography data on protein crystal would be necessary to understand this phenomena thoroughly. The wild-type R100 *merR* including all the other mutants were cloned in pKK223-3 for overproduction and purification, and the specific DNA-protein binding experiments were carried out as in the accompanying report.

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