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Overproduction and Operator DNA-Protein Blotting of R100 Mutant MerR from *Shigella flexneri*

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Wild-type and four mutant R100 *merR* genes were cloned and the proteins overproduced under *tac* promoter control of pKK223-3. His118Ala, Cys117Ser, Cys126Ser, and wild-type MerR were successfully overproduced although amino-terminal 14 amino acids deletion mutant MerR was not successful. The amount of overproduced wild-type MerR protein as well as other mutant MerR was between 15%-20% of the total protein. The protein was able to be purified up to 95% homogeneity. Specific DNA-protein blotting experiments showed that the 95 bp operator containing DNA fragment could bind to Cys126Ser, His118Ala, and wild- type MerR, but not to Cys117Ser. These results were consistent with the previously reported complementation experiment results that His118Ala, Cys126Ser, and wild-type MerR could repress the *mer* operon but Cys117Ser could not.

The most thoroughly investigated plasmid-mediated heavy metal resistance system is the conferring of the resistance to mercuric ions (4, 12, 16, 18). Similar systems have been found on plasmids isolated from many types of bacteria (16, 18). Mercury resistance determinant encodes mercuric reductase necessary for the enzymatic reduction of Hg^{2+} to Hg^{0} which volatilize. The mercuric reductase gene is a part of a well regulated *mer* operon, where additional genes are involved in regulation of the system and in mercury transport (4, 16, 18). The organization of Gram-negative mercury resistance systems of R100 (*Shigella*), Tn*501* (*P. aeruginosa*), and pDU1358 (*Serratia*) are similar (Fig. 1). DNA sequence and minicell analysis indicated that the Hg^{2+} resistant system is composed of many polypeptides: MerR, MerP, MerT, MerC, MerA, and MerB (4, 16, 17, 18). Wild-type MerR protein from gram-positive *Bacillus* RC607 was shown to bind to the divergent operator-promoter sequence and regulate mercury resistance both positively and negatively (12, 13). In the previous report, it was shown that His 118Ala, Cys126Ser, wild-type MerR could repress R100 *mer* operon, but Cys117Ser and the amino-terminal deletion mutants could not. In this report, wild-type and mutant MerR were overproduced, purified, and tested for specific operator binding ability to perceive the cause of previously reported repressions of R100 *mer* operon by them.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids

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

Growth Media and Growth Conditions

JM105 harboring appropriate plasmid or phage was grown on M9 medium (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 50 μM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) (14) supplemented with 0.2% glycerol and thiamine (10 μg/ml) at 37°C. For the overproduction of MerR protein, JM105 harboring appropriate plasmid was grown on Luria Broth medium (LB). The medium was supplemented with ampicillin (100 μg/ml) as required.

Overproduction and Purification of MerR

To overproduce R100 MerR and mutant R100 MerR, JM105 harboring appropriate plasmid was grown at 37°C in shaking incubator until optical density at 600 nm reached 0.5, at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. Cells were harvested 2.5 h later by centrifugation at 6000×g and resuspended in 30 ml of buffer A (100 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5% glycerol, 2 mM $CaCl_2$). The resuspended bacteria were passed through a prechilled French press cell twice at 5000 lb/in. pressure. The lysates were centrifuged in SS34 rotor at 20 k rpm

for 45 min at 4°C. The pellet was resuspended in ice-cold buffer A, and kept on ice for 20 min. The resuspended protein samples was centrifuged in SS34 rotor at 20 k rpm for 10 min at 4°C. Supematant was collected and centrifuged again in SS34 rotor at 20 k rpm for 40 min at 4°C. The resulting pellet was resuspended in 1.25 ml of buffer B (buffer A containing 0.5 M NaCl) and kept on ice for 10 min. The dissolved protein samples was centrifuged in SS34 rotor at 18 k rpm for 20 min. SDS-polyacrylamide gel electrophoresis of the supematant usually indicated that 60%~85% of the protein was in the 16-kDa band. Glycerol was added to 30% and samples were stored at -20°C. For further purification, protein samples from above were precipitated by the addition of ammonium sulfate to 50% saturation and the precipitate collected by centrifugation in SS34 rotor at 20 k rpm for 20 min at 4°C. The pellet was suspended in buffer C (buffer A containing 0.1 M NaCl) and applied to heparin sepharose CL-6B (Pharmacia) previously equilibriated in the same buffer. After washing with 4 column volumes of buffer C, the protein was eluted with a 0.1 to 1.5 M NaCl gradient in Buffer A. Wild-type MerR and mutants were eluted between 0.4 and 0.5 M NaCl and were more than 95% pure.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein samples were resuspended in a loading buffer (62 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.007% bromophenol blue) and heated to 100°C for 4 min and electrophoresed in a 18% polyacrylamide-SDS gel electrophoresis system as described (14).

Preparation of Probes

DNA probes used in the specific DNA-protein blotting was prepared in the same way as primer extension, in principle, involving M13 templates. M13 SS DNA from TM012 which contained the 94 bp operator fragment (Table 1) was primer extended from the 17-mer oligonucleotide primer for M13 using the Klenow fragment (3 U), 2 μl of a dATP-dGTP-dTTP solution (10 mM of each), and 2 μl of [α-^{32}P] dCTP (800 Ci per mmol). The reaction was incubated for 20 min at 37°C then chased with 1 U Klenow fragment and 1 μl deoxyribonucleotide solution (0.5 mM each of dATP, dGTP, dCTP, and dTTP) for 15 min at 37°C. The mixture was heated for 10 min at 65°C to inactivate the Klenow fragment. An 94 bp fragment containing whole operator-promoter region was removed by cleavage with *Eco*RI and *Hin*dIII for 45 min at 37°C. The fragment separated by electrophoresis through a 5% polyacrylamide gel (1:30 acrylamide:N, N'-methylene-bis-acrylamide linkage) at 200 volts in 1X TBE buffer (14). Following electrophoresis, the gel was covered with plastic wrap and autoradiographed for 5 min with Kodak XAR film. The radioactive probe was excised from the gel, crushed in a microcentrifuge tube, and eluted with 400 μl of 0.1X SSC for 4 h in a shaking incubator. The tube was centrifuged for 30 sec at 12,000 x g to pellet the polyacrylamide. The DNA was ethanol precipitated from the supematant ovemight at -70°C then resuspended in 50 μl of TE buffer.

DNA-Protein Blotting Procedure

Specific DNA-protein blotting was performed by the method described by Silva *et al.* with a slight modification (15). Electrophoresis of the total cellular protein was carried out on SDS-18% polyacrylamide gel as described in Materials and Methods. Transfer of the protein to nitrocellulose paper for DNA-protein blotting was done after the electrophoresis. The gel was immersed in 500

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant genotype or description	Reference or source
E. coli strains		
JM105		
	△(*lac-proAB*) *thi strA* *endA sbcB15 hsdR4* (F'*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Z*△*M15*)	8
Phages		
M13mp18, M13mp19	Templates for DNA sequencing	19
TM012	94 bp R100 operator-promoter fragment (559-652 in Fig. 2) in *Eco*R1-*Hin*dIII sites of M13mp19	10
Plasmids		
R100	Hg^r plasmid from *Shigella flexneri*	10
pKK223-3	Overexpression vector with *tac* promoter	Pharmacia LKB
pKPY1000	R100 *merR* (wild-type) in pKK223-3	This study
pKPY1001	R100 mutant (Csy117Ser) in pKK223-3	This study
pKPY1002	R100 mutant (Csy126Ser) 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

ml of western transfer buffer (3 g tris, 14.4 g glycine, 250 ml methanol in 1000 ml of deionized H_2O). The same size of nitrocellulose paper (BA85, Schleicher and Schuell) was cut and "western blot sandwich" was prepared. Electroblot was done at 100 volt at 4°C overnight. After the electroblot, "the sandwich" was disassembled and the nitrocellulose paper was immersed in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.25% gelatin, 0.1% NP-40). The buffer containing the paper was shaken gently at room temperature for 1 h. 1% Non-fat milk was added to the 200 ml of new NET buffer and the paper was kept in the new NET buffer for the additional 1 h to reduce non-specific binding of the operator fragment. The paper was then placed in a plastic pouch, and 10^5 cpm/ml [^{32}P] probe in 200 ml of standard binding buffer (1 mM Na-EDTA, 10 mM Tris-HCl, pH7.0, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl pyrollidone) was added. BSA, Ficoll, and polyvinyl pyrolidone were included in the binding buffer because they reduced non-specific sticking of DNA to nitrocellulose filters. The pouch was incubated with the DNA probe for 60 min at room temperature to allow DNA binding. During incubation, the pouch was occasionally agitated. The binding reaction was terminated by removing the nitrocellulose paper from the pouch, and washing it in 150 ml of the standard binding buffer for 60 min with three or four changes of the buffer, and afterwards placed on a Kimwipe to dry. The dried nitrocellulose paper was exposed to XAR-5 film overnight at -70°C.

RESULTS AND DISCUSSION

Overproduction and Purification of R100 MerR and Various R100 MerR Mutants

JM105 harboring an appropriate plasmid (Table 1) were grown at 37°C and induced with 1 mM IPTG for 2~3 h to overproduce wild-type and mutant R100 MerR. Cells were lysed with a French press as described previously in Materials and Methods. Protein samples from the above isolation protocol were electrophoresed in a 18% polyacrylamide-SDS gel electrophoresis system (Fig. 3). Protein molecular weight marker (MW-SDS-70L from Sigma Co.) was used to show molecular weights in kDa (Fig. 3, lane 1). When cells containing pKPY1000 were not induced, or induced just for 20 min, there was no overproduced MerR visible at 16-kDa region (Fig. 3, lane 2, 3 respectively). when pKPY1000 was induced for 2 h or 3 h, overproduced MerR band at 16-kDa region was clearly visible in both experiments by coomassie brilliant blue staining (Fig. 3, lane 4, 5 respectively). The amount of overproduced MerR protein was typically between 15%~20% of total protein. The top two bands at around 29-kDa marker were overproduced β-lactamase from pKK223-3 vector which has β-lactamase gene (*bla*) located at down stream of the multicloning site of the

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                          proposed Helix-Turn-Helix
                           <-------- -------->
                         *   *    ** ** * * * **   *       * *
pI258                MGMKISELAKACDVNKETVRYYERKGLIAGPPRNESGYRIYSEET 45
RC607                MKFRIGELADKCGVNKETIRYYERLGLIPEPERTEKGYRMYSQQT 45
Tn501           MENNLENLTIGVFAKAAGVNVETIRFYQRKGLLLEPDKPYGSIRRYGEAD 50
R100            MENNLENLTIGVFAKAAGVNVETIRFYQRKGLLREPDKPYGSIRRYGEAD 50
pDU1358         MEKNLENLTIGVFAKAAGVNVETIRFYQRKGLLPEPDKPYGSIRRYGEAD 50

                  *  * *    * * * **  *      *   *         *
pI258           ADRVRFIKRMKELDFSLKEIHLLFGVVDQDGERCKDMYAFTVQKTKEIER 95
RC607           VDRLHFIKRMQELGFTLNEIDKLLGVVDRDEAKCRDMYDFTILKIEDIQR 95
Tn501           VTRVRFVKSAQRLGFSLDEIAELLRL  EDGTHCEEASSLAEHKLKDVRE 98
R100            VVRVKFVKSAQRLGFSLDEIAELLRL  DDGTHCEEASSLAEHKLKDVRE 98
pDU1358         VTRVRFVKSAQRLGFSLDEIAELLRL  DDGTHCEEASSLAEHKLQDVRE 98

                *   * *    *  *   *        ** *  *
pI258           KVQGLLRIQRLLEELKEKCPDEKAMYTCPIIETLMGGPDK     135 aa
RC607           KIEDLKRIERMLMDLKERCPENKDIYECPIIETLM   KK     132
Tn501           KMADLARMEAVLSELVCACHARRGNVSCPLIASLQGGASLAGSAMP 144
R100            KMADLARMETVLSELVCACHARKGNVSCPLIASLQGEAGLARSAMP 144
pDU1358         KMTDLARMETVLSELVFACHARQGNVSCPLIASLQGEKEPRGADAV 144

Percent identities 36/135=27% conserved in all 5 sequences
pI258      100%  59%  36%  35%  35%
RC607            100  36   37
Tn501                 100  94   88
R100                       100  88
pDU1358                         100
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Fig. 1. Amino acids sequence homology among different *merR*.
pI258 is from *S. aureus*, RC607 from *Bacillus* sp. RC 607 chromosome, Tn*501* from *Pseudomonas aeruginosa*, R100 from *Shigella flexneri*, and pDU1358 from *Serratia*, This was produced with the multiple alignment program of Feng and Doolittle (3) with a gap penalty of 8. The proposed conserved helix-turen-helix motif is marked. Astericks indicate residues conserved in all 5 sequences.

tac promoter (1, Fig. 3. in an accompanying paper). Since the run-off transcription from induced *tac* promoter continued to pass *merR* and β-lactamase gene, β-lactamase was also overproduced whenever pKK223-3 was induced by IPTG. All three mutants showed the same overproduced band at 16-kDa region except for pKPY 1004 (data not shown). plasmid pKPY1004 contained the amino-terminal 14 amino acids deletion mutant of R100 MerR resulting in loss of the putative helix-turn-helix (Fig.1). Failure to overproduce amino-terminal deletion mutant might be due to great unstability of the mutant. The extraction with buffer B was performed to increase the purity after a French press as described in Materials and Methods and SDS-polyacrylamide gel electrophoresis indicated that usually 60~85% of the protein was R100 MerR corresponding to the 16-kDa position (Fig. 3, lane 6). Further purification was carried out with heparin sepharose CL-6B (Pharmacia) column as described in Materials and Methods. The R100 MerR protein was almost 95% pure (Fig. 3, lane 8).

Specific DNA-protein Blotting

In vitro MerR from *Bacillus* sp. RC607 binds as a transcriptional repressor in the absence of Hg^{2+} and as an activator in the presence of Hg^{2+} to a symmetrical region within the overlapping divergent promoters P_{TPCAD} and P_R of Tn*501*, resulting in repression and induction of *mer* structural gene expression respectively (5, Fig. 2). This area of dyad symmetry (7 bp repeats separated by 4 bp) lies between the -10 and -35 hexamers of P_{TPCAD} (Fig. 2). Both P_R and P_{TPCAD} have extensive homology with the consensus *Escherichia coli* σ-70 promoter sequence although the spacing between the respective -10 and -35 region is unusally 19 bp long (Fig. 2). In *mer* of R100 from *Shigella flexneri*, the divergent transcripts originate 17 bp apart, and the untranslated 5′ end of *merR* mRNA overlaps the MerR binding site (10, Fig. 2). Outside of the region of dyad symmetry, R100 and Tn*501* differ by 20% in the untranslated 5′ end of the

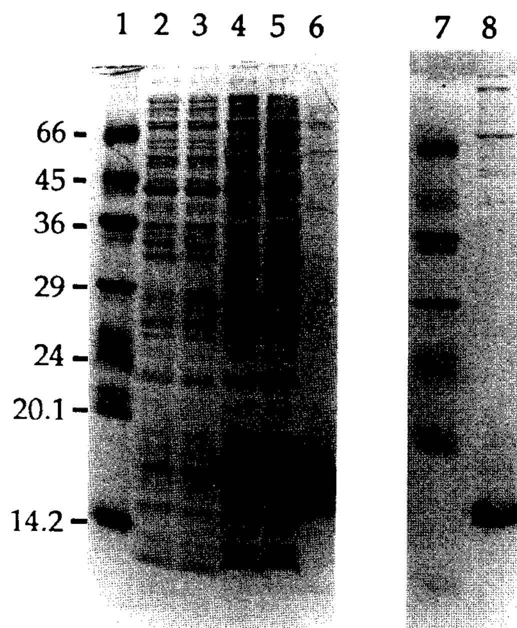

Fig. 3. Overproduction and purification of R100 wild-type MerR

To overproduce MerR, JM105 harboring pKPY1000 were grown at 37°C and induced with 1 mM IPTG for 2-3 h. Cells were lysed with a French press as described in Materials and Methods. Protein samples were electrophoresed in a 18% polyacrylamide-SDS gel electrophoresis system as described in Materials and Methods. Lanes: 1, protein low molecular weight marker shown in kDa; 2, cells harboring pKPY1000 were uninduced; 3, induced for 20 min; 4, induced for 2 h; 5, induced for 3 h; 6, after French press and extraction with buffer B; 7, the same protein molecular weight marker as that of lane 1; 8, after purification in heparin sepharose CL-6B (Pharmacia) column as described in Materials and Methods.

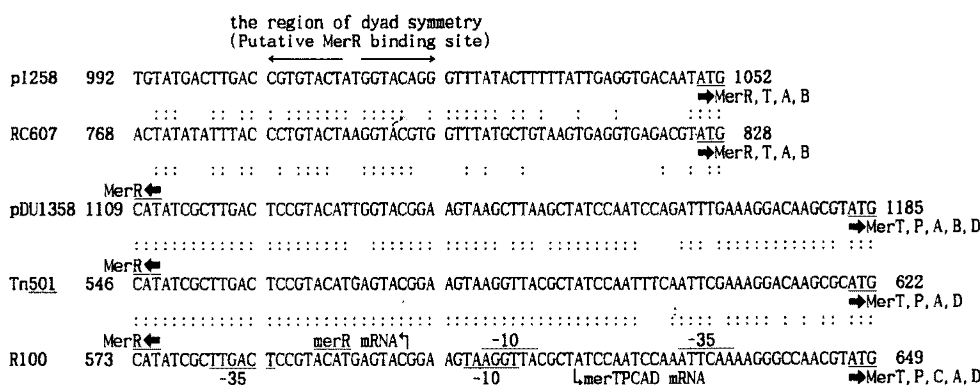

Fig. 2. Operator-promoter DNA sequence homology from the different microorganisms.

The proposed operator/promoter regions from the two Gram-positive systems and the three Gram-negative systems are shown. The Gram-negative system begin with the CAT (i. e. ATG) start codons for the divergently transcribed *merR* genes and end with the ATG start codons of the first structural gene, *merT*. pI258 is from *S. aureus*, RC607 from *Bacillus* sp. RC 607 chromosome, pDU1358 from *Serratia*, Tn*501* from *Pseudomonas aeruginosa*, and R100 from *Shigella flexneri*.

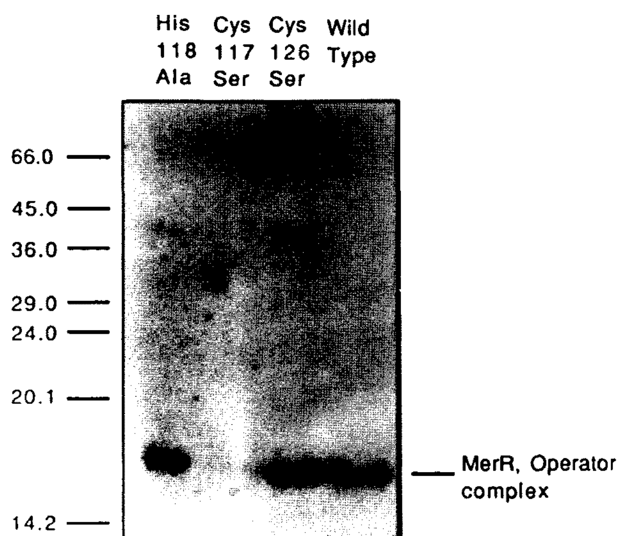

Fig. 4. Detection of the binding of R100 operator and promoter-containing DNA fragment to R100 MerR wild-type and mutants.

Total cellular Proteins were separated on an SDS-18% polyacrylamide gel as described in Materials and Methods. The protein were transferred onto a nitrocellulose filter, producing a replica of the original gel pattern, and renatured as described in Materials and Methods. His118Ala, Cys 126Ser, and wild-type MerR could bind to the operator fragment, but Cys117Ser could not. Low molecular weight marker was shown in kDa in the left.

structural gene transcript (10, 11). *In vivo* studies on the *mer* operon of Tn*501* show that none of the *mer* structural gene product is required for repression or transcription activation at the *mer* structural gene promoter (2, 7, 8).

In this report, the effect of various mutation on binding was shown by a specific DNA-protein blotting experiment (Fig. 4). After induction of JM105 harboring appropriate plasmids, all total proteins were separated in SDS-18% polyacrylamide gel and transferred to nitrocellulose paper and renatured. A 94 bp fragment containing whole R100 operator and promoter sequence (Table 1) was labelled by $[\alpha\text{-}^{32}P]$ dCTP and binding of the fragment to the renatured proteins was done as described previously. The results showed that the operator fragment could specifically bind to Cys126Ser, His118Ala, and wild-type MerR, but not to Cys117Ser (Fig. 3). The fragment did not bind to other proteins in the total cellular protein except the case of the Cys126Ser. The non-specific binding of the operator fragment to other cellular protein was noticed but this kind of binding was not observed in other cases even though all the four lanes contained the same amount of protein. These results were consistent with the previously reported complementation results (Fig 4. in the accompanying paper) which showed that His118Ala, Cys126Ser, and wild-type could repress R100 *mer* operon in the absence of mercuric ion but Cys117Ser could not. The site-directed mutation of Cys-117 to Ser might change the conformation of putative operator binding helix-turn-helix structure greatly, so that it could not bind to operator sequence. Other possible explanation was that Cys117Ser might lose ability to form the MerR dimer necessary to actively bind to the operator. But Mutation at His 118, and Cys-126 did not impair the ability of mutant MerR protein to bind to the putative operator sequence. The two mutation might not decrease the binding ability of helix-turn-helix structure to the operator.

In conclusion, the nature of gene regulation of R100 *mer* operon from *Shigella flexneri* by R100 *merR* was studied with three site-directed R100 *merR* mutants and an amino-terminal deletion mutant. The mutants were purified up to 95% purity except the amino-terminal deletion mutant. The results of the specific DNA-protein blotting suggested that His118Ala, Cys126Ser, and wild-type R100 MerR could repress *mer* operon even in the absence of mercuric ion, and these results were consistent with those previously reported.

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