Selection and characterization of mercury-independent activation mutants of the Tn501 transcriptional regulator, MerR

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MerR is the transcriptional regulator of the mercury-resistance (*mer*) operon of transposon Tn501, acting at the *mer* promoter as both an activator in the presence of mercuric salts and a repressor in their absence. This paper reports a method for selection of constitutive activator mutants, which activate transcription in the absence of Hg^{II}, and the characterization of these MerR^{AC} proteins. At least two mutations in the MerR protein were found necessary for strong mercury-independent activation, and these mutations lie in the C-terminal two-thirds of the MerR protein near the Hg^{II}-binding cysteines. A triple mutation was shown to increase activation over the corresponding double mutations. All mutant proteins caused further activation in the presence of Hg^{II}. The data support a mechanism in which a conformational change of one or both MerR subunits in the homodimer drives a distortion of DNA bound to a helix–turn–helix structure in the N-terminal region. A mutation in this putative helix–turn–helix region severely reduced both the repressor and activator functions of MerR.

Keywords: repressor, activator, MerR family, transposon, mercury resistance

INTRODUCTION

MerR from Tn501 regulates expression of the mercury-resistance (*mer*) genes. In the absence of mercury it represses transcription of the mercury-resistance operon (*merTPAD*) from a single promoter, P_T, and in the presence of Hg^{II} it activates transcription several hundredfold from the same promoter (Lund & Brown, 1989a). It also maintains repression of its own promoter, P_R (Lund *et al.*, 1986). All these functions are carried out by a single MerR homodimer bound to the dyad symmetrical operator DNA between the –35 and –10 elements of P_T (O'Halloran & Walsh, 1987). Unlike most positively controlled promoters (Raibaud & Schwartz, 1984), both the –35 and –10 elements of P_T show good similarity to those of a canonical *Escherichia coli* σ^{70} promoter (5/6 and 3/6 respectively; Lund *et al.*, 1986). However, the constitutive promoter activity of P_T is very weak, due to the abnormally long spacer

sequence between the promoter elements (19 bp compared to 17 ± 1 bp in a typical promoter; Harley & Reynolds, 1987), which is essential to the normal induction of the promoter (Lund & Brown, 1989b; Parkhill & Brown, 1990). Both MerR and RNA polymerase remain bound to the promoter during induction and some of the contacts of MerR with the DNA at the centre of the operator are altered on induction (O'Halloran *et al.*, 1989; Frantz & O'Halloran, 1990; Heltzel *et al.*, 1990). Mutations in the RNA polymerase α and σ^{70} subunits can affect the expression of P_T, but the basis for this is not known (Caslake *et al.*, 1997) and, unlike a number of other activators, MerR does not contact site I of the α -subunit (A. Ansari *et al.*, unpublished data, quoted in Ishihama, 1993).

These observations led to a model for P_T activation in which MerR, on binding Hg^{II}, directly causes a distortion of the DNA at the promoter, realigning the –10 and –35 elements and facilitating open complex formation by RNA polymerase (Lund & Brown, 1989a; O'Halloran *et al.*, 1989). O'Halloran and co-workers have shown that the addition of Hg^{II} salts to the MerR–DNA complex caused unwinding by 33° and straightening of a MerR-induced bend in the operator

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DNA (Ansari *et al.*, 1992, 1995). This DNA distortion is due to MerR-dependent induction as shown by a mercury-independent activation mutant of MerR *in vitro* (Parkhill *et al.*, 1993). The MerR protein contains an N-terminal element predicted to form a helix–turn–helix motif (Dodd & Egan, 1990) responsible for DNA binding, and four C-terminal cysteine residues, three of which are involved in tridentate binding of one Hg^{II} ion per dimer (Ross *et al.*, 1989; Shewchuk *et al.*, 1989b; Wright *et al.*, 1990; Utschig *et al.*, 1995).

The model for regulation by MerR predicts that there should be two conformations of the protein: a repressor conformation which binds to DNA such that the –10 sequence cannot be contacted by RNA polymerase and the open transcriptional complex cannot be formed; and an activator conformation in which Hg^{II} is bound to the C-terminal region of MerR and distorts the DNA, allowing RNA polymerase contacts to be made and transcription from P_T to be initiated.

In the absence of structural data for MerR, evidence for the existence of two conformations of MerR can be obtained by isolating constitutive activator and constitutive repressor mutants. Ross *et al.* (1989) isolated mutants of Tn21 MerR with altered repression and activation phenotypes, and Parkhill *et al.* (1993) reported the construction of a strong mercury-independent activator mutant of Tn501 MerR, which in the absence of Hg^{II} caused the same distortion of *mer* operator DNA as did the wild-type Hg^{II}–MerR complex. Comess *et al.* (1994) generated a similar mutant (in a synthetic *merR* gene) combining A89V and S86C changes. The formal possibility exists that these few mutations directly affect the interaction of MerR with RNA polymerase or with DNA and activate the DNA without changing the conformation of MerR. We therefore attempted to isolate a series of mutant MerR proteins to test further the two-conformation hypothesis for the action of MerR.

In this paper we describe the isolation by random and site-directed mutagenesis of constitutively activating MerR mutants and the characterization of the mutants obtained. We also report a mutation affecting DNA binding by MerR. The hypothesis that Hg^{II} acts in promoter regulation by altering the equilibrium between two conformational states of MerR, one repressing and one activating, is supported by distribution of the mutations in the protein and by the increasing activation observed within a single–double–triple mutation series and by the further activation seen with all mutants in the presence of Hg^{II}.

METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. They were grown in LB (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing 0.2% glucose, or in supplemented M9 minimal medium (Miller, 1972) containing 0.2% glucose, thiamin (20 µg ml⁻¹) and 0.2% Casamino acids. Dehydrated media were purchased from Becton Dickinson or LabM. Antibiotics were used at the

following concentrations: carbenicillin (Cb), 250 µg ml⁻¹; chloramphenicol (Cm), 35 µg ml⁻¹; kanamycin sulphate (Kn), 50 µg ml⁻¹; trimethoprim lactate (Tp), 25 µg ml⁻¹; tetracycline (Tc), 15 µg ml⁻¹ (except as described in the text when used for assaying the expression of P_T::Tc^r fusions). X-Gal (Sigma) was used at a concentration of 34 µg ml⁻¹.

Plasmids and vectors. Plasmids used in this study are listed in Table 1. A reporter plasmid, pMU-HT108, containing a P_T::*lacZ* transcriptional fusion, was constructed. This was generated by initially cloning a 108 bp *HpaII*–*TaqI* fragment containing the *merOP* region from pJOE114 (Brown *et al.*, 1983) into the *AccI* site of pUC19 (Yanich-Perron *et al.*, 1985). A recombinant plasmid with the insert in the desired orientation was selected and the insert was subcloned as a *BamHI*–*HindIII* fragment upstream of the promoterless *lacZ* gene in the low-copy-number IncW plasmid pMU2385 (Praszkier *et al.*, 1992), such that transcription of the *lacZ* gene was driven by the P_T promoter. A second P_T transcriptional fusion (P_T::*tetC*) was available in plasmid pBR-EA-1 (Lund & Brown, 1989b), which confers on cells resistance to high levels of tetracycline only when the promoter P_T is induced (e.g. in the presence of a plasmid expressing wild-type MerR and inducing concentrations of HgCl₂).

To provide the *merR* gene *in trans* to these *tetC* and *lacZ* reporter plasmids, a Kn^r Tc^s derivative of plasmid pACYC184 (Chang & Cohen, 1978) was constructed. pACYC184 has the p15 origin of replication and is therefore compatible with both pBR-EA-1 and pMU-HT108, but a Tc^s derivative was required to allow detection of the Tc^r status of pBR-EA-1. The Kn^r gene and promoter from pBGS18^r (Spratt *et al.*, 1986) were used to provide a selectable marker for the pACYC184 derivative; these were isolated as a 1.5 kb *MluI* fragment, the 5' overhangs were filled in with Klenow DNA polymerase, and the fragment was cloned into the *HincII* site of pUC19. An insert of the desired orientation was recloned as a *BamHI*–*HindIII* fragment between the *BamHI* and *HindIII* sites of pACYC184, removing the promoter and 5' end of the pACYC184 Tc^r gene in the process. The resulting plasmid (pACK15) was confirmed to be Cm^r Kn^r Tc^s. The individual wild-type and mutant (A89V, S131L and A-S) *merR* genes (Parkhill *et al.*, 1993) were cloned into this vector at the *EcoRI* site in the Cm^r gene, so that the *merR* gene was expressed from the constitutive chloramphenicol acetyltransferase promoter. These plasmids were designated pACK-90-16, pACK-A89V, pACK-S131L and pACK-A-S, respectively.

pACYC184-based plasmids containing mutated *merR* genes were constructed by cloning mutagenized DNA fragments from the vectors pMa90-16, pMcA89V and pMcS131L into pACYC184 or pACK15. These were designated pAC-*mut* and pACK-*mut*, respectively, where *mut* refers to the mutation in MerR, as determined by sequence analysis.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim, and were used according to the manufacturers' instructions. Klenow DNA polymerase was purchased from Amersham and Sequenase from United States Biochemical Corporation (USB). NEN DuPont supplied [γ -³²P]ATP. Chemical reagents were obtained from Fisons, Sigma or Boehringer Mannheim and were analytical grade wherever possible.

DNA manipulations. DNA manipulations were carried out largely as described by Sambrook *et al.* (1989). Preparative electrophoresis for purification of DNA fragments was carried out on a 6% or 8% (30:1 cross-linking) polyacrylamide gel in

Table 1. Bacterial strains and plasmids used in this study

<i>E. coli</i> strain	Genotype	Reference
CSH26 Δ recA	<i>ara</i> Δ (<i>lac-pro</i>) <i>thi</i> Δ recA	Miller (1972)
WK6	Δ (<i>lac-pro</i>) <i>galE</i> <i>strA</i> F' [<i>lacI</i> ^a Z Δ M15 <i>proA</i> ⁺ B ⁺]	Stanssens <i>et al.</i> (1989)
WK6 <i>mutS</i>	As above but <i>mutS</i> 215::Tn10; Tc ^r	Stanssens <i>et al.</i> (1989)
Plasmid	Properties	Reference
pACYC184	Cloning vector with p15a origin of replication; Cm ^r Tc ^r	Chang & Cohen (1978)
pAC- <i>mut</i>	pACYC184 with <i>Eco</i> RI fragment encoding a mutant MerR cloned into Cm ^r gene; Tc ^r	This study
pAC-90-16	pACYC184 with <i>Eco</i> RI fragment encoding wild-type MerR cloned into Cm ^r gene; Tc ^r	This study
pACK15	pACYC184 with Kn ^r gene replacing Tc ^r gene; Cm ^r Kn ^r	This study
pACK- <i>mut</i>	pACK15 with <i>Eco</i> RI fragment encoding a mutant MerR cloned into Cm ^r gene; Kn ^r	This study
pACK-90-16	pACK15 with <i>Eco</i> RI fragment encoding wild-type MerR cloned into Cm ^r gene; Kn ^r	This study
pBGS18+	Derivative of pUC19 with Kn ^r gene replacing Cb ^r ; Kn ^r	Spratt <i>et al.</i> (1986)
pBR-EA-1	Derivative of pBR322 in which the Tc ^r gene is expressed from P _T ; Cb ^r	Lund & Brown (1989b)
pJOE114	Tn501::pBR322 fusion; Cb ^r	Brown <i>et al.</i> (1983)
pKK223.3	Vector for IPTG-inducible protein expression; Cb ^r	Amann <i>et al.</i> (1983)
pKK- <i>mut</i>	Derivative of pTO90-16 containing a mutant <i>merR</i> gene for overexpression of mutant MerR; Cb ^r	This work
pMa/c5-8	Mutagenesis vectors; Cb ^r /Cm ^r	Stanssens <i>et al.</i> (1989)
pMa90-16	MerR fragment from pTO90-16 cloned into pMa5-8	Parkhill <i>et al.</i> (1993)
pMcA89V, pMcS131L	Derivatives of pMa90-16 bearing site-specific mutations in <i>merR</i> ; Cm ^r	Parkhill <i>et al.</i> (1993)
pMU2385	Single-copy promoter probe vector with promoterless <i>lacZ</i> gene; Tp ^r	Praszkier <i>et al.</i> (1992)
pMU-HT108	pMU2385 with P _T transcribing the <i>lacZ</i> gene; Tp ^r	This study
pRZH-2	Promoter probe vector with P _T :: <i>lacZ</i> fusion; Kn ^r	Lund <i>et al.</i> (1986)
pRZH-18	Promoter probe vector with P _T :: <i>lacZ</i> fusion; Kn ^r	Lund <i>et al.</i> (1986)
pTO90-16	MerR overexpression construct derived from pKK223.3; Cb ^r	O'Halloran & Walsh (1987)
pUC19	Cloning vector; Cb ^r	Yanisch-Perron <i>et al.</i> (1985)

a mini-Protein II gel system (Bio-Rad). DNA was stained with ethidium bromide, visualized briefly under UV illumination, eluted by crushing the excised polyacrylamide fragment and soaking for several hours in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% SDS, 0.1 mM EDTA) and then purified by phenol extraction and ethanol precipitation.

Hydroxylamine mutagenesis. Plasmid DNA (2–10 µg) was incubated in 100 µl 50 mM sodium pyrophosphate (pH 7.0), 100 mM NaCl, 2 mM EDTA, 1 M hydroxylamine at 70 °C. Aliquots (25 µl) were removed at 15, 30, 60 and 120 min and added to 1 ml precipitation solution (150 mM sodium acetate, 20 µg tRNA ml⁻¹, 50% propan-2-ol). The DNA was recovered by centrifugation at 12000 g for 15 min, and the pellet was washed with 70% ethanol before being dried under vacuum. The DNA was resuspended in 50 µl Tris/HCl (10 mM, pH

7.5), 1 mM EDTA, and used in transformation of recipient *E. coli* cells. For selection of non-activating mutants from plasmid pAC-90-16, the strain *E. coli* CSH26 Δ recA(pRZH-18) (Lund *et al.*, 1986) was transformed and transformants were selected on minimal medium containing HgCl₂ (1.0 µg ml⁻¹) and Cb, Tc and X-Gal at the concentrations given above. For selection of activating mutations from plasmids pACK-90-16, pACK-A89V and pACK-S131L, the strain *E. coli* CSH26 Δ recA(pMU-HT108, pBR-EA-1) was used and transformed cells were plated onto supplemented minimal media containing Cb, Kn, Tp, X-Gal (at the concentrations given above) and Tc (5 or 10 µg ml⁻¹).

DNA sequencing. DNA sequence analysis was by the chain termination method (Sanger *et al.*, 1977) using modified T7 DNA polymerase, or with Sequenase (USB) using the modified Sanger protocol supplied by the manufacturer. Both reactions

were performed on single-stranded templates generated from the mutagenesis vector pMa/c5-8 by superinfection, as described by Stanssens *et al.* (1989). Oligonucleotide sequencing primers were complementary to the end of the multiple cloning site of pMa/c5-8, or to sequences within the *merR* gene.

Site-directed mutagenesis. This was performed using the gapped-duplex method of Stanssens *et al.* (1989). The *merR* gene was cloned into the *EcoRI* site of pMa/c5-8 and mutagenized using the following oligonucleotides: D78N (5'-GGGTGCCATICTCCAGCCG), E77K (5'-TGCCATCCTTCAGCCGCAG), E77K-D78N (5'-TGCCATTCTTCAGCCGCAG), L74Q (5'-TCCAGCCGCTGCAGCTCGG), P127L (5'-GCGATCAGCAGGCAGGAAA), where the underlined letters show the nucleotides altered from the wild-type *merR* gene.

β -Galactosidase assays. These were carried out as described by Miller (1972). Strains containing the plasmid(s) to be assayed were grown overnight in supplemented M9 medium in the presence of appropriate antibiotics. The cultures were then diluted 1:50 into fresh medium and grown with shaking at 37 °C until the OD₆₀₀ was between 0.4 and 0.8. If the strains were to be induced with Hg^{II}, HgCl₂ was added to a final concentration of 1.8 µM 1 h before assaying the culture. All assays were carried out in triplicate.

Purification of MerR proteins. Wild-type MerR was purified according to a modification of the protocol described by Parkhill *et al.* (1993). Cells containing pTO90-16 (for wild-type MerR) or isogenic derivatives containing mutant *merR* genes (the pKK-*mut* series) were grown in 800 ml LB, 0.2% glucose with shaking at 37 °C to OD₆₀₀ = 0.7, isopropylthiogalactoside was added to 50 µM, and growth continued for a further 1 h. Cells were harvested by centrifugation and resuspended at 0.5 g ml⁻¹ in buffer A (100 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 10% glycerol, containing protease inhibitors). Cells were disrupted by sonication on ice for a total of 8 min in 30 s bursts separated by a cooling period of 2.5 min. The sonicate was centrifuged (24000 g, 30 min, 4 °C) and the supernatant was discarded. The pellet was washed with 20 ml buffer A and centrifuged again. The pellet was then suspended in buffer Z (20 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 1 M NaCl equal to the original volume of buffer A used. The suspension was incubated for 5 min at 4 °C and centrifuged (24000 g, 30 min, 4 °C). Streptomycin sulphate was slowly added to the supernatant to a final concentration of 0.2% and the suspension was incubated for 30 min at 4 °C before the precipitate was collected by centrifugation (24000 g, 30 min, 4 °C) and discarded. Ammonium sulphate was slowly added to the supernatant to 50% saturation and the precipitate was allowed to form over 30 min at 4 °C. The precipitate was collected by centrifugation as before and redissolved in 10 ml buffer Z/1 M NaCl (for 800 ml culture).

The protein solution was diluted 10-fold with buffer Z and loaded onto a heparin sulphate column pre-equilibrated in buffer H (20 mM sodium phosphate, pH 6.0, 0.1 M NaCl, 0.1 mM EDTA, 2 mM DTT, 10% glycerol). The column was washed with buffer H and eluted with a gradient of 0.1–0.5 M NaCl in buffer H. Fractions containing the highest concentrations of MerR were identified by SDS-PAGE, made 50% in glycerol and stored under nitrogen at -20 °C. To minimize oxidation of cysteine residues, all buffers were purged with nitrogen gas, filtered through 0.45 µm filters and then degassed before use.

Protein concentrations were measured by the method of Bradford (1976) on a routine basis. These were standardized against the Lowry method to provide comparability with concentrations determined in other studies (Shewchuk *et al.*, 1989c; Frantz & O'Halloran, 1990; Parkhill *et al.*, 1993; Comess *et al.*, 1994), as the Bradford assay gives an apparent sevenfold lower concentration for MerR than does the Lowry method when using bovine serum albumin as a standard.

Gel retardation assay. DNA fragments following *EcoRI*/*Bam*HI digestion of 2 µg pRZH-2 DNA (Lund *et al.*, 1986) were end-labelled by incubation with [γ -³²P]ATP (3000 Ci µmol⁻¹; 37 TBq µmol⁻¹) and T4 polynucleotide kinase, according to the manufacturer's protocol. The end-labelled 200 bp *EcoRI*-*Bam*HI fragment containing the *mer* operator was purified by electrophoresis on 8% acrylamide gels as described above, but the fragment was visualized by brief autoradiography. The DNA concentration was calculated from the known starting concentration of DNA and by following incorporated radioactivity.

Aliquots of the purified DNA fragment (1 fmol) were incubated with increasing concentrations of protein. Protein-DNA complexes were formed in 20 µl incubation buffer [10 mM Tris/HCl pH 8, 100 mM potassium glutamate, 0.1 mM EDTA, 1 mM CaCl₂, 5% (v/v) glycerol, 1 mM DTT and 100 µg BSA ml⁻¹] for 1 h at 10 °C. HgCl₂ was present at 0.75 µM where required. Following incubation the complexes were resolved on 6% (37.5:1) native polyacrylamide gels which were pre-run at 300 V for at least 45 min in a cold room. The samples were loaded on the gels and were then run at 300 V for 2.5 h.

DNA fragments were visualized using a Molecular Dynamics phosphorimager and the intensities of fragments of different mobility were analysed with the Imagequant software. The fraction of radioactivity in the band that represents specific protein-DNA complexes was plotted against concentration of the respective proteins on a semi-logarithmic graph. The apparent K_D in such a plot is the concentration of the protein at half-maximal binding.

RESULTS

Selection of activating MerR mutants

Previous selection procedures using a single copy of P_T linked to a selectable reporter had isolated only P_T promoter-up mutations, and single-step random mutagenesis of wild-type MerR had not revealed the desired strong mercury-independent activation mutants of MerR. The selection procedure based on two P_T gene fusions to independent selectable markers, *tetC* and *lacZ* (Fig. 1), should avoid the isolation of promoter-up mutations and allow infrequent mutations to be isolated. Colonies that were *tetC*⁺*lacZ*⁺ would be likely to contain activating mutations in the *merR* gene, rather than separate promoter-up mutations in the two P_T promoters.

The selection procedure was tested by transforming existing pACK-*merR* plasmids into *E. coli* CSH26- Δ *recA* (pMU-HT108, pBR-EA-1). The resulting transformants were tested for Tc^r in the presence and absence of mercury. The wild-type and single mutants (A89V or S131L) could grow on 15 µg Tc ml⁻¹ only in the presence of HgCl₂. The positive control strain expressing the

Fig. 1. Diagram showing the selection procedure for isolation of activating *merR* mutants. Constitutively activating mutations cause expression of tetracycline resistance and β -galactosidase activity. Details of the selection are given in the text.

constitutive activator MerR(A-S) (Parkhill *et al.*, 1993), however, could grow on $15 \mu\text{g Tc ml}^{-1}$ in the absence of HgCl_2 . In each case the expression of β -galactosidase corresponded with the expression of tetracycline resistance (i.e. colonies showing high-intensity blue colour were more tetracycline resistant than colonies showing lower intensity blue colour).

Each of the plasmids pACK-90-16 (wild-type), pACK-A89V and pACK-S131L (Table 1) was mutagenized with hydroxylamine, and used to transform *E. coli* CSH26- ΔrecA (pMU-HT108, pBR-EA-1). After 2–3 d incubation at 37°C on supplemented minimal media containing Cb, Kn, Tp, X-Gal and Tc (5 or $10 \mu\text{g ml}^{-1}$), blue colonies were picked and plated in replicates on the same medium with or lacking Tc ($10 \mu\text{g ml}^{-1}$), together with *E. coli* CSH26 ΔrecA (pMU-HT108, pBR-EA-1) containing either of the parent plasmids or pACK-A-S, as controls. Colonies that were tetracycline resistant and showed strong β -galactosidase activity were selected for further characterization. Seven such colonies were isolated using pACK-A89V as the parent plasmid and two using pACK-S131L; none was detected when pACK-90-16 was used.

Characterization of activating MerR mutants

The selected mutant colonies were grown in liquid culture and pACK-*merR* DNA was prepared by alkaline lysis. The 500 bp *merR*-containing *EcoRI* fragments were purified, cloned into pACYC184 (Chang & Cohen, 1978), and used to transform *E. coli* CSH26 ΔrecA -(pRZH-18) (Lund *et al.*, 1986). For each mutant a transformant was identified in which the *merR* fragment

in the recombinant plasmid was in the correct orientation to be transcribed from the CAT promoter. These transformants were assayed for β -galactosidase activity in the presence and absence of mercury (Fig. 2). This ensured that effects were entirely due to mutations within the *merR* gene and not to mutations elsewhere on the mutagenized plasmid or to induced changes in the host chromosome during the initial selection. In addition, each mutant *merR* gene was subcloned from the pACYC184 construct into M13mp19 in both orientations and fully sequenced to determine the site and number of mutations present in the gene. The positions of each mutation in the MerR protein are shown in Fig. 3.

Using MerR S131L as the parent, the additional mutations R62Q or V124A caused weak activating phenotypes, each with uninduced β -galactosidase activities about $10 \pm 7\%$ of the induced wild-type activity. This compares with the more strongly activating phenotype of the A89V-S131L mutant MerR of about $50 \pm 10\%$ and the S131L uninduced activity of $2.2 \pm 0.9\%$ of induced wild-type activity. When A89V was used as the parent, further mutations L74Q, E77K, G79V, G79S, T80P and P127L were separately isolated. The mutants containing A89V with G79D, G79S, or T80P had only weakly activating phenotypes in the absence of Hg^{II} ($4.3 \pm 1.5\%$, $4.8 \pm 1.6\%$ and $2.8 \pm 0.8\%$ of induced wild-type activity, respectively) and were induced in the presence of Hg^{II} to a level similar to that of the wild-type MerR. In the absence of Hg^{II} the difference between mutants containing these double mutations and that containing the A89V mutation alone ($1.4 \pm 0.5\%$ of wild-type induced activity) was small. Mutations containing A89V with L74Q, E77K, or P127L were more strongly activating in the absence of Hg^{II} ($39 \pm 17\%$, $48 \pm 5.7\%$ and $34 \pm 7.0\%$ of induced wild-type activity, respectively).

One strain was found to contain two mutations, E77K and D78N, in addition to the A89V mutation of the parent, and this triple mutation gave the most strongly activating phenotype of all ($86 \pm 17\%$ of induced wild-type activity). Each of the mutations was recreated as a single or double mutation using the procedure of Stanssens *et al.* (1989) on the plasmid pMa90-16 (Parkhill *et al.*, 1993), with the oligonucleotides described in Methods. Two other mutations which showed high Hg^{II} -independent induction when present with the A89V mutation, namely L74Q and P127L, were also recreated as single mutations in the *merR* gene. These mutant *merR* genes were sequenced and were also subcloned into pACYC184 for β -galactosidase assay as described above. The results of the β -galactosidase assays are shown in Fig. 2.

Of the single mutants, L74Q showed the strongest activation in the absence of Hg^{II} ($4.9 \pm 0.1\%$ induced wild-type activity), the remainder being E77K, $2.2 \pm 0.6\%$; D78N, $0.74 \pm 0.06\%$; P127L, $0.75 \pm 0.12\%$. The two newly created double mutants, A89V-D78N and D78N-E77K, mediated stronger Hg^{II} -independent ac-

Fig. 2. β -Galactosidase assays of *E. coli* CSH26 Δ recA(pRZH-18) strains carrying mutant activating *merR* genes in the presence (hatched) and absence (solid) of Hg^{II}. Each strain carries the *merR* gene cloned in pACYC184 and expressed from the CAT promoter. Each assay was carried out in triplicate and standard deviations are shown. All single mutants were created by site-directed mutagenesis, as were mutants D78N-E77K and A89V-D78N; the construction of A89V-S131L has been described (Parkhill *et al.*, 1993). All other mutants were obtained by random mutagenesis from either A89V or S131L as parent.

Fig. 3. Positions of activating mutations of Tn501 MerR. The putative helix-turn-helix and the mercury-binding cysteine residues are marked. Mutations R62Q and V124L (and A89V) show enhanced mercury-independent activation when present with S131L; the remaining mutations (including S131L) show enhanced mercury-independent activation when paired with A89V.

tivation than any of the single mutants ($26 \pm 4\%$ and $14 \pm 2\%$), although these levels were lower than those observed for the A89V-E77K or triple mutant. These compare with uninduced wild-type activities of $0.48 \pm 0.16\%$. All the single mutants had lower induced activities than the wild-type MerR, but only P127L (at $45 \pm 10\%$) showed an induced activity in the presence of Hg^{II} of less than 80% of the induced wild-type value. This poor induction in the presence of Hg^{II} was also seen in the A89V-P127L mutant ($55 \pm 11\%$).

DNA binding by constitutive activator mutant and wild-type MerR proteins

DNA-binding studies were carried out using a 200 bp DNA fragment containing the wild-type P_T operator region and different concentrations of the wild-type MerR protein or constitutive activator mutants. Gel retardation assays indicated that wild-type MerR binds the operator site with apparent K_D values of 2×10^{-10} M in the presence of Hg^{II} and 1×10^{-10} M in the absence of Hg^{II}. The binding affinities of the mutant proteins tested are shown in Table 2.

Table 2. Equilibrium dissociation constants of the mutant MerR proteins

Protein	K_D (M)	
	+ Hg	- Hg
Wild-type	1.9×10^{-10}	1.0×10^{-10}
A89V	1.7×10^{-10}	0.9×10^{-10}
D79N	1.9×10^{-10}	1.4×10^{-10}
E77K	4.2×10^{-10}	2.6×10^{-10}
A89V, D78N	2.9×10^{-10}	1.6×10^{-10}
A89V, E77K	1.6×10^{-10}	1.4×10^{-10}
D78N, E77K	3.3×10^{-10}	2.9×10^{-10}
A89V, D78N, E77K	1.4×10^{-8}	2.3×10^{-8}

These results indicate that the single mutations have little or no effect on DNA binding, a result which agrees with the *in vivo* data. The apparent K_D values obtained for the double mutants were also similar to wild-type levels. The triple mutant, however, bound the wild-type operator with a considerably lower affinity (K_D 1.4×10^{-8} M in the presence of Hg^{II} and 2.3×10^{-8} M in its absence). *In vivo* this mutant shows the strongest activation in the absence of Hg^{II} (80% of wild-type) but its interaction with the DNA is slightly stronger in the presence of Hg^{II} as tested *in vitro*. Thus the triple mutant appears to bind the DNA with approximately 100-fold lower affinity and the effect of Hg^{II} on K_D is reversed with respect to wild-type MerR binding.

Selection and characterization of poorly activating mutants

E. coli CSH26 Δ recA(pAC-90-16, pRZH-18) grows normally on L-agar containing Kn, Tc and X-Gal, but slowly, if at all, on the same medium supplemented with HgCl₂ ($1.0 \mu\text{g ml}^{-1}$). This may be due to the accumulation of toxic products of X-Gal hydrolysis (Park *et al.*, 1992; Ross *et al.*, 1989) when the promoter is induced,

and was exploited in the isolation of poorly activating mutants of MerR.

Following hydroxylamine mutagenesis of pAC-90-16 and transformation of *E. coli* CSH26 Δ recA(pRZH-18), colonies were isolated on L-agar containing Kn, Tc, X-Gal and HgCl₂ (1.0 µg ml⁻¹). Colonies were grown overnight in L-broth supplemented with Kn and Tc and tested for β -galactosidase activity. Four colonies which failed to induce β -galactosidase activity were identified and plasmid DNA was prepared from them. The *merR*-containing *Eco*RI fragment was isolated, cloned again into pACYC184 and the phenotype in *E. coli* CSH26- Δ recA(pRZH-18) was confirmed.

The *merR* genes from each of the four colonies were sequenced after cloning the *Eco*RI fragment into M13-mp18. Three of the genes contained the same mutation, a G to A transition at nucleotide 475 of Tn501, causing a R25H mutation in MerR; the remaining gene contained a termination codon at position 368 of Tn501 (resulting in a truncated MerR terminating at A60) and two further mutations in the non-translated downstream region of the gene. The induction of β -galactosidase activity by one of the three R25H mutants in the presence and absence of Hg^{II} was determined, and compared with isogenic control strains containing the wild-type *merR* gene (on plasmid pAC-90-16) and lacking *merR* (plasmid pACYC184). Repression and activation of P_T by Hg^{II} were both severely reduced in the strain containing the R25H mutant MerR, repression was not detectable, and induction was 2% of that conferred by wild-type MerR. This is consistent with R25 being in the recognition helix of the helix-turn-helix structure.

DISCUSSION

Mutants of MerR isolated in our selection procedure contained at least two mutations. It is not clear why this is so, as the site-directed single mutation L74Q gave a slightly higher mercury-independent β -galactosidase activity than did the selected double mutants A89V-T80P and A89V-G79D. Double mutations arising from the single parents A89V or S131L may have arisen more frequently than single mutations from the wild-type in our experiments, and the results may merely reflect the limited number of mutants characterized. Moreover, the β -galactosidase activity assayed in liquid culture does not directly reflect the accumulation of the indole chromophore (and expression of tetracycline resistance) in the plate selection procedures.

A notable feature of the selected mutations is their clustering in the MerR sequence. Inspection of the *merR* DNA sequence shows no significant changes in base composition which may predispose specific parts of the gene to mutagenesis. Several of the mutations are close to the Hg^{II}-binding cysteine residues. This is not surprising, as these mutations (when paired with A89V or S131L) may partially mimic the effects of Hg^{II} binding on MerR conformation and activity. Most of the

mutations that pair with A89V (namely L74Q, E77K, D78N, G79S, G79D and T80P) are close to C82, which is thought to be the sole Hg^{II}-binding cysteine in one MerR subunit of the active homodimer (Helmann *et al.*, 1990; Shewchuk *et al.*, 1989b) and is proposed to be close to the subunit interface (Shewchuk *et al.*, 1989b). Comess *et al.* (1994) isolated a A89V-S86C constitutive activator double mutant and showed that this mutant was able to distort the *mer* operator in the presence or absence of Hg^{II}. They suggested that the introduction of an additional cysteine may allow the formation of a new disulphide bond to lock the protein into an activating conformation; our data show that nearby mutations can cause activation without generating additional thiol groups.

Only two mercury-independent activating mutations arose from the S131L parent in this work. One (V124A) is a conservative substitution close to the Hg^{II}-binding cysteine C126. The other (R62Q) is not close to the Hg^{II}-binding cysteines in the linear sequence, but may be close to the subunit interface in this protein (Shewchuk *et al.*, 1989a) and therefore affect the activation phenotype.

The P127L mutation is of particular interest as the equivalent residue is conserved in all MerR proteins (data not shown) and it is adjacent to the Hg^{II}-binding C126. The A89V-P127L double mutant has a strongly activating mercury-independent phenotype. However, it shows only a slight additional activation in the presence of mercury, and the corresponding P127L single mutation confers a very weak mercury-dependent activation phenotype. The single mutation had previously been identified as a reduced activation mutant in the Tn21 MerR (Ross *et al.*, 1989). Summers and colleagues (Livrelli *et al.*, 1993) have shown by *in vivo* footprinting that the P127L mutant protein has an apparent altered interaction with the RNA polymerase-*mer* DNA complex and does not show a normal footprinting response to the addition of Hg^{II}. Thus, it is possible that the P127L mutation may hinder (but not abolish) the binding of Hg^{II} by C126, but be able to potentiate mercury-independent activation in association with A89V. The conserved sequence CP(I/L)I in all MerR proteins at positions equivalent to C126 to I129 (data not shown) may represent a turn in the MerR tertiary structure.

D78 is conserved in almost all MerR sequences and the D78N single mutation has little activating effect but does slightly reduce activation by Hg^{II}-MerR. When combined with other mutations, as in the A89V-D78N and E77K-D78N double mutants, the level of Hg^{II}-independent activation is higher, but is not as strong as the A89V-E77K or A89V-D78N-E77K mutants. The E77 and D78 acidic residues were targeted, along with two other pairs of acidic residues (D68/E69 and E83/E84), by Comess *et al.* (1994). In their study the E77 residue was changed to a glutamine rather than the lysine which we isolated by random mutagenesis. It was shown that the E77Q-D78N mutant had no effect by itself but did

contribute to a repression-deficient phenotype when paired with the E83Q-E84Q mutant. Our results show that the more drastic E77K mutation, especially in conjunction with the D78N and A89V changes, confers a stronger activating phenotype.

A full interpretation of the significance of the individual mutations will eventually require elucidation of the three-dimensional structure of the protein; but, in the absence of a structure, this study can contribute to an understanding of the mechanism of action of MerR. Our data can be rationalized in terms of a model in which MerR exists in two stable conformations when bound to DNA: a repressor conformation (the R state), which represses P_T ; and an activator conformation (the A state), which induces P_T and in which the binding of a single ion of Hg^{II} alters the equilibrium between the two forms. The mercury-independent mutations are hypothesized to alter the equilibrium in favour of the activator conformation (Parkhill *et al.*, 1993). The strongest support to date for this equilibrium model comes from the incremental mutation series (A89V, A89V-E77K and A89V-E77K-D78N), which shows increased mercury-independent activation through the series, yet further activation of all members of the series can be obtained on the addition of Hg^{II} . The other two series of single and double mutations, starting with D78N or E77K, show the same effect. We suggest that each mutation slightly displaces the equilibrium in favour of the activator conformation. Within the limits of error of our experiments, wild-type MerR in the presence of Hg^{II} may be almost fully in the activator conformation, as only a further 10% activation over the wild-type induced level of β -galactosidase expression was detected in our experiments.

The apparent K_D values for the binding of wild-type MerR to DNA (2×10^{-10} M in the presence of Hg^{II} and 1×10^{-10} M when Hg^{II} is absent) are comparable with the results of Parkhill *et al.* (1993). The majority of activating mutants in this and in previous studies (Comess *et al.*, 1994) have shown a similar affinity for the operator in the absence of Hg^{II} . The exception was the strongly activating MerR(A-S) mutant studied by Parkhill *et al.* (1993), which had a lower affinity for the operator. In all cases the binding of Hg^{II} reduced the affinity for the operator DNA. The difference in binding energy of MerR and MerR- Hg^{II} to DNA has been postulated to reflect the free energy required for distortion of the DNA helix (Ansari *et al.*, 1992; O'Halloran *et al.*, 1989). In the studies reported here, the strongest constitutive activator mutant (A89V-D78N-E77K) has a significantly lower affinity for the operator than wild-type MerR, and DNA binding is strengthened slightly in the presence of Hg^{II} . We can interpret these data on a model in which the cumulative mutations displace the structure of the MerR protein towards an activating conformation, but simultaneously destabilize the interaction with the operator. In the case of the triple mutation, the binding of Hg^{II} may be required to provide structural stability to MerR and therefore strengthen the protein-DNA interaction.

The mutation R25H has properties consistent with a defect in the DNA-binding activity of this protein, as both the repression and activation ratios conferred by this protein are much lower than in the wild-type. This mutation provides further evidence that the helix-turn-helix structure predicted to be present between residues I10 and R29 is involved in DNA binding. Our data are consistent with those which indicate that two separate regions of the protein are involved in the binding of DNA and of Hg^{II} , and in which the mutation E22K reduces DNA binding *in vitro* about 100-fold (Shewchuk *et al.*, 1989a).

The phenotypes of the parental mutations A89V and S131L reported here differ from those of the identical mutations in Tn21 (Ross *et al.*, 1989). Both single mutations give rise to higher levels of uninduced expression *in vivo* in Tn21 (24% and 128% of wild-type induced activity) than in Tn501 (both about 5% of wild-type induced activity). Some of these effects may be due to the differences in protocols for measuring β -galactosidase activities (e.g. measuring activity *in cis* or *in trans*, different host strains, different reporter constructs). However, the differences may also be due to the nine amino acids which differ between the two MerR proteins (Barrineau *et al.*, 1984; Foster & Brown, 1985; Misra *et al.*, 1984). These are at L34(R), T52(V), R55(K), E77(D), A107(T), R121(K), G135(E), S137(G) and G140(R), where the equivalent Tn21 residues are given in parentheses. These residues are formally equivalent to multiple mutations in the other protein and one or more of them may give rise to the differences in properties of the wild-type and mutant MerR proteins from Tn501 and Tn21.

The mutations described here support and provide further evidence for a conformational model for activation of the *mer* genes by MerR. The MerR dimer binds symmetrically to the dyad sequence (the *mer* operator) in P_T , one subunit to each operator half-site, and represses transcription from the promoter. On binding Hg^{II} across the subunit interface in the C-terminal region of the protein, a conformational change in the MerR dimer occurs, which results in distortion of the DNA at the centre of the operator, through the binding interactions of the each subunit of MerR to each operator half-site on the DNA, and this activates the promoter.

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