Tn5 Insertion Mutations in the Mercuric Ion Resistance Genes Derived from Plasmid R100

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The mercuric resistance (*mer*) genes of plasmid R100 were cloned into plasmid pBR322. A series of transposon Tn5 insertion mutations in the *mer* genes were isolated and mapped. The mutants were characterized phenotypically by their sensitivity to Hg^{2+} and by binding and volatilization of $^{203}Hg^{2+}$. Dominance and complementation tests were also performed. Mutations affecting the previously described *mer* genes *merR* (regulation), *merT* (transport), and *merA* (reductase) were characterized. Evidence was obtained for two new *mer* genes, which have been called *merC* and *merD*. A restriction enzyme map of the *mer* region was drawn with the gene order *merRTCAD*. Transcriptional *merR-lac* and *merA-lac* fusions were generated by insertion of phage Mu d *amp lac* into plasmid R100-1. These were used to study regulation of *mer* gene expression. The *merR* gene product appears to regulate negatively its own expression as well as acting as both a negative and a positive regulator of the *merTCA* genes.

Plasmid-encoded resistance to mercuric ions is widespread in both gram-positive and gram-negative bacteria (9, 32, 35, 37). Resistance involves the reduction of Hg^{2+} to elemental Hg, which volatilizes from the medium (34). The enzyme that catalyzes this reaction is the intracellular mercuric reductase, a flavin adenine dinucleotide-containing multimeric protein that uses NADPH as a cofactor (33). Expression of the reductase system requires induction by subinhibitory concentrations of Hg^{2+} (14, 34). Another component of the resistance mechanism is an Hg^{2+}-specific transport function that carries Hg^{2+} into the cytoplasm, where it is detoxified by the reductase enzyme. The transport function was inferred from the hypersensitivity to Hg^{2+} of strains defective in reductase. Hypersensitivity was correlated with inducible hyperbinding of Hg^{2+} (14, 26), suggesting that an inducible transport function was operating.

The mercuric resistance (*mer*) region of plasmid R100 was investigated with mutations generated by inserting Tn801 (14) and with cloned DNA fragments (26). Three *mer* genes were identified and mapped (14): *merR*, which codes for a diffusible regulatory element; *merT*, which encodes the transport function; and *merA*, which specifies the mercuric reductase subunit. A model explaining the control of expression of the *mer* genes was proposed (14, 26, 35). It is thought that the *merT* and *merA* genes are expressed coordinately in an operon controlled by the positively acting regulatory protein speci-

fied by *merR*. In addition, the *merR* product is thought to act as a repressor in uninduced cells (14). After induction, the repressor is converted into a positively acting inducer of transcription of the *mer* operon.

Several Hg^{2+}-inducible polypeptides associated with the *mer* region have been visualized in maxicell and minicell systems (12, 18, 19). A polypeptide with a molecular weight of approximately 60,000 was shown to be the reductase subunit (12, 30, 33). Polypeptides of 15,000, 14,000, and 12,000 daltons are associated with *mer* genes located proximal to *merA* (18). The 12,000-dalton protein is cytoplasmic and is rapidly processed from a 13,000-dalton molecule. The 15,000- and 14,000-dalton proteins are associated with the cytoplasmic membrane and are thus candidates for the transport system (18, 19).

Although we refer to the plasmid as R100 in this report, derivatives of the same plasmid have been carried over the last 25 years under the names R222 and NR1. In fact, the source of the cloned *mer* region used here was NR1 (22), whereas our previous mapping work was with R100-1 (14). We know of no difference pertinent to the current work.

In this paper, we report the mapping and properties of a series of Tn5 insertion mutations in the cloned *mer* genes derived from plasmid R100. Most mutations were assigned to known *mer* genes, but some allowed tentative identification of two new *mer* genes. Studies on the

regulation of expression of *mer* genes by using *mer-lac* fusions are also reported.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Tables 1 and 2. Further details of Tn5 insertion mutations are given below.

Media and chemicals. Medium components were from Oxoid Ltd., unless stated otherwise. LB agar, M9 salts minimal medium, lambda base agar, lambda top agar, LB, and tryptone broth were as described (23). LabLemco agar used for some disk and resistance level tests was described previously (15).

Antibiotics and other chemicals were from Sigma Chemical Co. or were the best grade available from British Drug House unless stated otherwise. Drugs were used at the following concentrations: ampicillin (Beecham Research Laboratories, Ltd.), 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 20 μg/ml; nalidixic acid, 20 μg/ml; tetracycline, 10 μg/ml; trimethoprim (Burroughs Wellcome Co.), 30 μg/ml.

Restriction endonucleases. Restriction enzymes and T4 ligase were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim Corp. and were used according to the instructions of the suppliers.

Complementation tests. Transposon insertion mutations in *mer* genes have been classified into two complementation groups, one representing mutations in *merR* and the other representing insertions elsewhere in the operon (14). Complementation tests were performed by the phenocopy mating techniques described before (14) when the plasmids to be tested were both R100-1 derivatives. For compatible plasmids (e.g., R100 and pUB5572 derivatives or R100 and pBR322 derivatives), stable heterozygous cells were constructed by conjugation and tested for mercury resistance (Hg^r) by the disk method.

Plasmid constructions. The in vitro manipulations of the *mer* genes are shown in Fig. 1 and described below. The *mer* region of plasmid NR1 was cloned in the ColE1::TnA vector RSF2124 by Miki et al. (22). Plasmid pRR134 carries R100 *Eco*RI fragment H and expresses hypersensitivity to Hg^{2+} (26). Resistance to Hg^{2+} is conferred by plasmid pRR130, which has both *Eco*RI fragments H and I (22). The *mer* genes were subcloned from these plasmids to reduce the size of the cloned DNA fragment harboring Hg^{2+} resistance and also to inactivate the IS*1* sequence, which is adjacent to *mer* in pRR130 and pRR134 and which is likely to promote deletions at high frequency (29). The *mer* genes of pRR130 reside on a 7.5-kilobase (kb) *Pst*I fragment. Cloning this *Pst*I fragment inactivates IS*1*. The plasmid which was used for most of our genetic studies (pDU1003) was derived from pRR130 in three steps (Fig. 1). First, *Pst*I-cleaved pRR130 DNA was mixed with *Pst*I-cleaved pBR322 DNA and ligated. One tetracycline-resistant (Tc^r) Hg^r ampicillin-sensitive (Ap^s) transformant (pDU962) carried the 7.5-kb *Pst*I *mer* fragment and an additional 4.5-kb *Pst*I fragment from pRR130. pDU962 was cleaved with *Eco*RI and ligated to remove a 2-kb *Eco*RI fragment. At the same time, the orientation of the *mer*-carrying *Eco*RI fragments was reversed with respect to the vector to generate pDU994. Subsequent cleavage with *Pst*I and

ligation removed a 3-kb *Pst*I fragment to yield pDU1003 (11.3 kb).

The small recombinant *mer* plasmid pDU1003 was cleaved with a variety of restriction enzymes. Only *Eco*RI and *Hin*cII cut within *mer* (Fig. 2). The following enzymes did not cut in *mer*: *Sal*I, *Sma*I, *Sac*I, *Xba*I, *Xho*I, *Pvu*II, *Bcl*I, *Bst*EII, *Hin*dIII, *Bam*HI, *Cla*I, *Bgl*II, *Acc*I, and *Ava*I.

The 7.5-kb *Pst*I *mer* fragment from plasmid pRR130 was also cloned directly into the low-copy-number vector pUB5572, replacing an 1,100-base pair (bp) *Pst*I fragment. One trimethoprim-resistant (Tp^r) Hg^r plasmid (pDU989) was retained for further study.

The proximal part of the *mer* region carried by plasmid pRR134 was on a 3.5-kb *Pst*I fragment that was cloned into pUB5572 (Fig. 1). Tp^r transformants were selected and scored for Hg^{2+} hypersensitivity by testing for failure to grow on trimethoprim agar containing 1 μg of HgCl$_2$ per ml. One clone, pDU991 (Fig. 1) was kept for subsequent analysis.

Deletion mutations were generated in vitro by removing DNA between the *Hin*cII sites in the *mer* region of plasmid pDU1003 to generate plasmids pDU995 and pDU1214 (Fig. 2). A partial *Hin*cII digest of pDU1003 DNA was fractionated on an agarose gel, and partial digest products were excised, ligated, and transformed into C600. Transformants were screened for loss of the 3,300-bp *Hin*cII fragment, the 3,100-bp *Hin*cII fragment, or both. One mutant (pDU995) had lost both fragments; another (pDU1214) had lost the distal 3,100-bp *Hin*cII fragment. A *Bam*HI-*Pst*I fragment of pDU1214 was inserted into *Bgl*II-*Pst*I–cleaved pUB5572 (Tp^r) to generate pDU1215, thus lowering the copy number of the *mer* genes of this derivative.

One *Pst*I fragment of pDU1003 *mer*::Tn5 plasmids carries *mer* sequences (including *merR* and promoter-proximal segment of the *mer* operon) lying between the *Pst*I site in IS*1* and the *Pst*I site in the inverted repeat of the inserted Tn5 element (31). Seven pDU1003 *mer*::Tn5 (*merR*$^+$) plasmids were used to generate derivatives carrying various amounts of the *mer* operon (but missing *merA*) by cloning the *merR*-containing *Pst*I fragment into the Tp^r vector pUB5572. In addition, two pDU1003 *merD*::Tn5 mutations were cloned into pUB5572 by the same technique. Tp^r transformants were selected in C600 carrying pDU3321, a *merR* mutant of pDU202. When the

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
C600	*thr leu lac supE*	N. Kleckner
XAcSu$^-$	Δ(*lac-pro*)XIII *ara argE rpoB gyrA thi*	J. Beckwith via N. Kleckner
DU5110	*lac his gyrA rpsL malA* Mu	Mu lysogen of DU1040; reference 13
DU5003	Δ(*lac-pro*)XIII *thi rpoB*	10
DU5111	Δ(*lac-pro*)XIII *thi rpoB* Mu	Mu lysogen of DU5003; this study
MAL103	Δ(*lac-pro*)XIII *rpsL* Mu *cts* d1(Ap^r *lac*)	6
ED2030	Δ(*lac*)XI74 *trp recA56 gal*	16

TABLE 2. Plasmids

Plasmid	Phenotypic markers	Comments	References
pDU202	Cm^r Sm^r Su^r Hg^r	Tc^s mutant of R100-1	17
pDU3321	Cm^r Sm^r Su^r Hg^s Ap^r	*merR*::Tn*801* mutant of pDU202	14
pDU3324	Cm^r Sm^r Su^r Hg^s Ap^r	*merR*::Tn*801* mutant of pDU202	14
pDU3316	Cm^r Sm^r Su^r Hg^{ss} Ap^r	*merA*::Tn*801* mutant of pDU202	14
pDU1186	Cm^r Sm^r Su^r Hg^{ss} Ap^r Ka^r	pDU3316 tagged with Tn*5*	14
pDU1124	Cm^r Sm^r Su^r Hg^{ss} Ap^r	*merA*::Mu d *amp lac* insert in R100-1	This study
pDU1135	Cm^r Sm^r Su^r Hs^s Ap^r	*merR*::Mu d *amp lac* insert in R100-1	This study
pDU1161-1170	Cm^r Sm^r Su^r Ka^r Ap^r Hg^s	R100-1 *mer*::Tn*5 merA*::Mu d *amp lac*	This study
pDU1171-1178	Cm^r Sm^r Su^r Hg^s Ka^r	R100-1 *mer*::Tn*5*	This study
pBR322	Ap^r Tc^r		4
pACYC184	Cm^r Tc^r		7
pUB5572	Tp^r IncW	Derived from R388, 8 copies per chromosome	8
pRR130	Ap^r Hg^r ColE1$^+$	ColE1::Tn*A* with *Eco*RI fragments H and I of R100	22, 26
pRR134	Ap^r Hg^{ss} ColE1$^+$	ColE1::Tn*A* with *Eco*RI fragment H of R100	22, 26
pDU989	Tp^r Hg^r IncW	*mer* genes cloned from pRR130 into pUB5572	This study
pDU1188	Tp^r Hg^{ss} Ka^r IncW	*merA*::Tn*5* mutant of pDU989	This study
pDU991	Tp^r Hg^{ss} IncW	Proximal part of *mer* operon cloned from pRR134 into pUB5572	This study
pDU1187	Tp^r Hg^{ss} Ka^r IncW	pDU991 tagged with Tn*5*	This study
pDU962	Tc^r Hg^r	*mer* genes cloned from pRR130 into pBR322	This study
pDU1003	Tc^r Hg^r	*mer* genes cloned from pRR130 into pBR322	This study
pDU1190	Tc^r Hg^{ss} Ka^r	*merA*::Tn*5* insertion into pDU962	This study
pDU995	Tc^r Hg^s	*mer* deletion mutant of pDU1003	This study
pDU1214	Tc^r Hg^r	pDU1003 with 3,100-bp *Hinc*II fragment deleted	This study
pDU1215	Tp^r Hg^r	pUB5572 with cloned *Pst*I-*Bam*HI fragment of pDU1214	This study
pDU1189	Ap^r Hg^{ss} Ka^r ColE1$^+$	*merA*::Tn*5* mutant of pRR130	This study
pDU1031-pDU1093	Tc^r Hg^s or Hg^{ss} Ka^r	*mer*::Tn*5* insertions in pDU1003	This study
pDU1125	Cm^r Hg^r	*mer$^+$* *Hind*III fragment cloned from pDU202 into pACYC184	This study
pDU1126	Cm^r Hg^s Ap^r	*merR*::Tn*801* mutation cloned from pDU3321 into pACYC184	This study
pDU1127	Cm^r Hg^s Ap^r	*merR*::Tn*801* mutation cloned from pDU3324 into pACYC184	This study
pDU1179-80	Tp^r Hg^s	Δ*merTCAD* deletions derived by cloning from pDU1003 *mer*::Tn*5* into pUB5572	This study
pDU1181-2	Tp^r Hg^{ss}	Δ*merCAD* deletions derived by cloning from pDU1003 *mer*::Tn*5* into pUB5572	This study

desired *merR$^+$*-containing fragment was present, the *merR* lesion of pDU3321 was complemented, and the cells with both plasmids were Hg^r. Tp^r Hg^r Tc^s kanamycin-sensitive (Ka^s) colonies were retained, and the pUB5572-*mer* recombinant plasmids were separated from pDU3321 by transformation. Their physical structures were then checked by restriction enzyme analysis.

To facilitate accurate physical mapping of the Tn*801* insertions in plasmids pDU3321 and pDU3324 (pDU202 *merR*::Tn*801* mutants [14]), the entire *mer* operon segments along with the inserted transposon were cloned on a *Hind*III fragment into the *Hind*III site in the Tc^r gene of pACYC184. (Tn*801* and the *mer* sequences do not have *Hind*III sites). Chloramphenicol-resistant (Cm^r) Ap^r Tc^s transformants were checked for their ability to complement pDU3316, a *merA* mutant of pDU202.

The 10-kb *Hind*III fragment of pDU202 (which is

equivalent to *Hind*III fragment 4 of R6-5 [36]) carrying the complete *mer* operon was cloned into pACYC184. In this case, Cm^r transformants were selected and checked for a Hg^r Tc^s phenotype. One (pDU1125) was selected for further study.

Isolation of *mer*::Tn*5* insertion mutations. Cultures of XAcSu$^-$-carrying pDU1003 *mer$^+$* or pDU991 *merA* were grown to late exponential phase in LBM broth and were infected with λ467 (*rex*::Tn*5 O*am29 *P*am80 *c*I837) at a multiplicity of 0.5 phage per cell. After incubation for 60 min at room temperature, the infected cells were spread on kanamycin agar to select for Tn*5* transpositions. Pools of >1,000 colonies were made in sterile saline. The cells were diluted into 20 ml of broth and were incubated for 18 h at 37°C. Plasmid DNA was isolated by the small-scale technique and used to transform C600. Ka^r transformants had Tn*5* inserted in plasmids. The colonies were replica plated onto Hg^{2+} agar. Cells with derivatives of pDU991
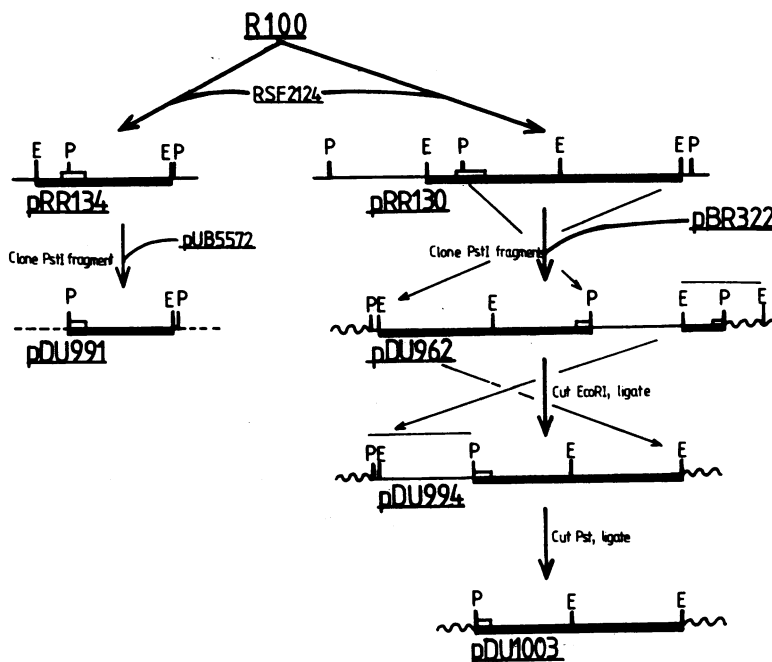
FIG. 1. Derivation of recombinant *mer* plasmids. The in vitro manipulations performed in the isolation of the recombinant *mer* plasmids illustrated here are described in the text. The generation of plasmids pRR130 and pRR134 was described previously (22). For the recombinant plasmids, the thin horizontal lines represent vector RSF2124 DNA, and the thicker line shows DNA cloned from the *mer* region of plasmid R100. The open boxes above the line show the location of all or part of IS*1*. The subcloning of these sequences into pBR322 (wavy lines) and pUB5572 (dashed lines) is also shown. The thin horizontal lines above the maps of pDU962 and pDU994 show the deleted DNA sequences. Restriction endonuclease cleavage sites for *Pst*I (P) and *Eco*RI (R) are labeled.

(*merA*) which had lost hypersensitivity were selected on agar with 1 µg of HgCl$_2$ per ml. pDU991 *merR*::Tn5 mutations were identified by their failure to complement pDU3321 (*merR*). Mutants of pDU1003 were identified by replica plating on 10 µg of HgCl$_2$ per ml. Only one mutant was retained from each transposition pool.

Mapping *mer*::Tn5 and *mer*::Tn*801* insertions. Transposon Tn5 is not cleaved by *Eco*RI but has *Hind*III and *Hinc*II sites located 1,200 and 190 bp, respectively, from the outer ends of its inverted repeats (20). Insertions in plasmid pDU1003 were first assigned to either the large (*mer* operator proximal) or small (*mer* operator distal) *Eco*RI fragment (Fig. 2). Then the sizes of the junction fragments formed by digestion with *Eco*RI + *Hind*III and by cleavage with *Hinc*II were measured. In this way, the position of the Tn5 insertion relative to both the *Eco*RI and the *Hinc*II sites in *mer* was determined. A similar procedure was used for mapping Tn5 insertions in pDU991.

The in vitro-constructed pACYC184 *mer* plasmid pDU1125 *mer*$^+$ and the cloned *merR*::Tn*801* mutations derived from plasmids pDU3321 and pDU3324 (14) were cleaved with *Eco*RI to determine the orientation of the inserted *Hind*III fragment with respect to the vector. Since the orientations of the Tn*801* insertions were known (14), it was possible to map the Tn*801* insertions by measurement of the *Hinc*II-generated junction fragments.

Construction of *mer-lac* fusions. The Mu d *amp lac* phage described by Casadaban and Cohen (6) was used to generate transcriptional *mer-lac* fusions in plasmid R100-1. Two classes of fusions were sought, one in *merR* and the other in *merA*. Strain DU5003 carrying R100-1 was infected with a fresh heat-induced lysate of MAL103, which carries Mu d *amp lac* phage particles. Ap^r transductants were selected at 30°C, and pools of ca. 10,000 colonies were made in broth. These were diluted to about 5 × 10^7 cells per ml and were incubated at 30°C for 4 h to obtain exponentially growing cells, which were mated with DU5110 (nalidixic acid resistant [Na^r] Mu *c*I$^+$) for 2 h at 37°C. Transconjugants with Mu d *amp lac* inserted in R100-1 were selected on ampicillin-nalidixic acid agar. These were replica plated on agar containing 10 µg of HgCl$_2$ per ml to identify Hg^s derivatives. Putative *mer*::Mu d *lac* insertion mutants were streaked on MacConkey agar with or without 0.1 µg of HgCl$_2$ per ml to identify Hg^{2+}-inducible or constitutive expression of the *lac* genes.

One mutant (plasmid pDU1135), which conferred Hg^{2+} sensitivity but which failed to complement pDU3321 (*merR*) and did complement pDU3116 (*merA*) and which gave constitutive expression of Lac, was thought to have a *merR-lac* fusion. Another (pDU1124), which conferred Hg^{2+} hypersensitivity and failed to complement pDU3116 (*merA*), but did complement pDU3321 (*merR*) and expressed Lac only
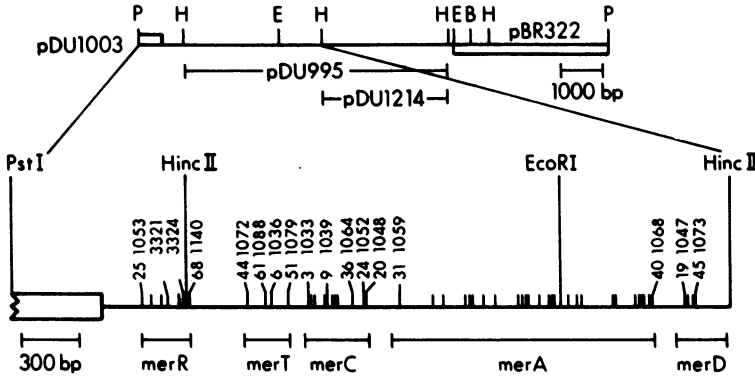
FIG. 2. Physical map of the *mer* region of plasmid pDU1003. The upper part shows a restriction map of pDU1003. The open box at the left represents the remnant of IS*1*; the open box at the right shows pBR322 vector sequences. Restriction endonuclease cleavage sites are labeled for *Pst*I (P), *Hinc*II (H), *Eco*RI (E), and *Bam*HI (B). The lines just below this map show the extents of the deletions in pDU995 and pDU1214. The lower diagram shows an expanded map of the *mer* region with sites of Tn*5* and Tn*801* (3321, 3324) insertions marked by vertical lines. The four-number code represents the four-digit number assigned to the pDU series plasmids; the two-number code represents the allele number of the Tn*5* insertions. The location of *mer* genes marked by these insertions are indicated by horizontal lines below the map.

after induction with Hg^{2+}, was thought to have a *merA-lac* fusion.

Transfer of *mer*::Tn*5* mutations from multicopy plasmids to a *merA*::Mu d *amp lac* fusion derivative of R100-1. Tn*5* insertions located in the *merR*, *merT*, and *merD* genes of plasmids pDU991 (Tp^r) and pDU1003 (Tc^r) were transferred by homologous recombination to pDU1124, a derivative of R100-1 having an insertion of Mu d *amp lac* in *merA* (Fig. 3). The Tn*5*-carrying mutant plasmids were transformed into DU5111 carrying the *lac* fusion plasmid pDU1124. The heterozygotes were mated with DU5110 (Na^r Lac$^-$), and Ka^r Na^r exconjugants were selected. These colonies were replica plated onto tetracycline or trimethoprim agar to identify exconjugants which had not inherited the parental *mer*::Tn*5* plasmid. The majority (ca. 95%) of Ka^r exconjugants retained the Tc^r or Tp^r marker, indicating that the nonconjugative vector plasmid had been mobilized into the recipient, presumably owing to formation of cointegrates by a single recombination event between the homologous *mer* sequences. The Ka^r Tc^s and Ka^r Tp^s colonies were tested for sensitivity to Hg^{2+}. The *mer-lac* plasmid pDU1124 conferred hypersensitivity to Hg^{2+} owing to the Mu d *amp lac* insertion in *merA*. Insertion of Tn*5* in the *merR* or *merT* genes of this plasmid resulted in loss of hypersensitivity owing to either direct inactivation of the transport function (*merT*::Tn*5*) or failure to induce the *mer* operon (*merR*::Tn*5*). Between 50 and 90% of the exconjugants retained the Hg^{2+} hypersensitivity phenotype and had presumably acquired Tn*5* insertions by transposition at other sites in the plasmid. The putative *mer*::Tn*5* homogenotes were then tested for the presence of *merR*$^+$ by complementation tests with pDU3321 (*merR*). This test was done to ensure that the appropriate *mer* gene was inactivated by the desired homogenotization event and that Tn*5* had not transposed to another site in *mer* to eliminate hypersensitivity. The putative homogenotes were also tested for Ap^r and for their Lac phenotype on MacConkey agar.

Those which retained the Mu d *amp lac* insertion (Fig. 3, crossover 1) were Ap^r Lac$^+$ (the Lac$^+$ phenotype being due to low-level constitutive expression of β-galactosidase). When the crossover was distal to Mu d *amp lac*, a Lac$^-$ Ap^s homogenote was formed (Fig. 3, crossover 2). In the case of *merD* mutants, the R100-1 *merA*$^+$ *merD*::Tn*5* recombinant was sought by selecting for Cm^r Ka^r Ap^s Tc^s exconjugants.

Measurement of sensitivity to mercuric ions. Three methods were used to assess the sensitivity of *mer*
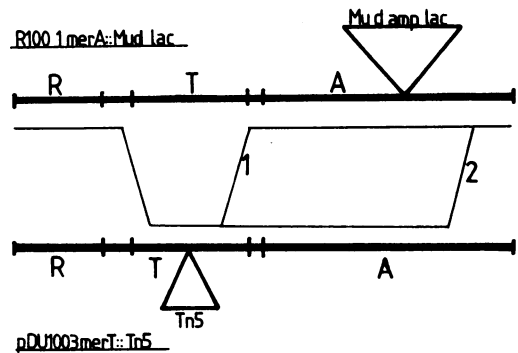

FIG. 3. Recombination between *mer*::Tn*5* insertion mutations and plasmid R100-1 *merA*::Mu d *amp lac*. The diagram shows the two classes of recombinants selected in crosses between pDU1003 *mer*::Tn*5* mutants and R100-1 *merA*::Mu d *amp lac*. This example shows a *merT*::Tn*5* insertion. The same principle applies to *merR* and *merC* insertions. The large triangles show the sites of Tn*5* and Mu d *amp lac*. R, T, and A represent the *merR*, *merT*, and *merA* genes, respectively. Crossover 1 generates a *merT*::Tn*5* *merA*::Mu d *amp lac* double mutant; crossover 2 results in a *merT*::Tn*5* *merA*$^+$ recombinant.

plasmid-bearing strains to Hg^{2+}: (i) toothpick streaks on Hg^{2+}-containing agar, (ii) disk tests, and (iii) colony resistance level determinations. In all cases, an antibiotic selective for the plasmid was incorporated in the agar. This was particularly important when testing plasmids which conferred Hg^{2+} hypersensitivity because loss of the plasmid allowed cells to grow at higher Hg^{2+} concentrations and could mask expression of hypersensitivity. If the plasmid encoded Tp^r, it was necessary to use M9 salts plus Casamino Acids medium, otherwise, LB or LabLemco agar was employed. Toothpick streaks on agar containing 1 or 10 μg of $HgCl_2$ per ml allowed the three Mer phenotypes to be distinguished; failure to grow on 1 μg/ml indicated hypersensitivity, whereas growth on 10 μg/ml was only achieved by resistant strains. Sensitive strains grew on 1 μg/ml but were inhibited by 10 μg/ml. In disk tests, plates were flooded with 10^{-2} or 10^{-3} dilutions of LB broth cultures. After drying, a 6-mm-diameter disk incorporating 10 μg of $HgCl_2$ was placed on the surface, and the plates were incubated for 18 h at 37°C. The diameter of the zone of growth inhibition was measured (37). Resistance levels were determined as previously described (14).

Isolation of plasmid DNA. A small-scale purification technique was employed for experiments involving routine screening and plasmid transformation. A sample of cleared lysate (0.5 ml) from a 10-ml broth culture was extracted with phenol, precipitated with ethanol, and passed through a small Sephadex G-50 column. DNA for in vitro manipulation was purified by dye buoyant density gradient centrifugation (21).

β-Galactosidase assays. Early exponential-phase LB cultures were induced by the addition of 0.5 to 5 μM $HgCl_2$ 30 min before sampling for the β-galactosidase assays, which were performed as described (11, 23).

Mercury volatilization. Mercury volatilization assays were performed as described (33), except that cells were suspended in sodium or potassium phosphate buffer (pH 7.0) with no added sodium azide. All assays contained 5 μM Hg^{2+}.

Mercury binding experiments. Cultures were grown to 50 to 70 Klett turbidity units (Klett colorimeter with no. 54 filter) in Oxoid tryptone broth. Then they were induced by the addition of 0.25 μM Hg^{2+} and incubated for a further 60 min. The cells were centrifuged and resuspended in tryptone broth. The binding of $^{203}Hg^{2+}$ was measured as described previously (26), except that tetracycline (100 μg/ml) was used instead of chloramphenicol to inhibit induction of uninduced cultures during the course of the assay. Samples were filtered on Whatman GF/C 2.4-cm glass fiber filters and washed twice with 5 ml of broth, and the bound radioactivity was measured by liquid scintillation spectroscopy.

RESULTS

Characterization of recombinant plasmids. The *mer* genes derived from plasmid R100-1 were cloned in pBR322, pUB5572, and pACYC184 vectors as described above and shown in Fig. 1. It had been reported previously (25) that resistance to Hg^{2+} did not increase when *mer* gene number increased, despite the fact that there was a gene-dosage effect for reductase activity assayed in broken-cell extracts. It should be noted that this increased level of reductase was not apparent when volatilization from whole cells was assayed (25; Table 3). The Hg^r phenotypes of the *mer*⁺ plasmids used in this study are shown in Table 3. Each of these plasmids except pDU1003 and pDU1125 expressed the same level of resistance to $HgCl_2$ as pDU202 (i.e., R100-1) did in tests for disk sensitivity, resistance level, and efficiency of plating. Despite their failure to express wild-type resistance, these multicopy *mer*⁺ plasmids synthesized elevated levels of reductase (Table 3). Indeed, they expressed higher levels than multicopy *mer* plasmids with normal Hg^r phenotypes.

Hypersensitivity to mercuric ions conferred by *merA* mutant plasmids. The Hg^{2+} hypersensitivity phenotype characteristic of *merA* mutant

TABLE 3. Properties of strain C600 with recombinant *mer*⁺ plasmids carrying the wild-type R100 *mer* operon

Plasmid	Resistance to $HgCl_2$		Efficiency of plating[a]		Mercuric reductase activity[b] (μmol of Hg^{2+} per min per g)	
	Inhibition zone (mm)[c]	Resistance level (μg/ml)	Uninduced	Induced	Whole cells	Broken cells
pDU202	0	10	$<10^{-4}$	1.0	22	9
pDU989	0	11	$<10^{-4}$	1.0		
pRR130	0	9	$<10^{-4}$	1.0	19	38
pDU962	0	8	$<10^{-4}$	1.0	23	40
pDU1003	6	3	$<10^{-4}$	10^{-3}	19	111
pDU1125	6	3	$<10^{-4}$		22	53
None	10	0.5	$<10^{-8}$	$<10^{-8}$	<0.02	<0.02

[a] Colony numbers on nutrient broth agar with 10 μg of $HgCl_2$ per ml compared with colony numbers without $HgCl_2$.

[b] Reductase activity was measured with preinduced cells at low substrate concentration (5 μM), which tends to lessen the difference between pRR130 and pDU202, previously reported to be sevenfold (25).

[c] Disk test zone diameter minus the diameter of the disk (6 mm).

plasmids is displayed best by disk tests with agar incorporating an antibiotic to select for retention of the plasmid (14). For this reason, some plasmids carrying *merA* lesions (pDU991 and pDU3316) were tagged with Tn5 so that their hypersensitivity phenotypes could be directly compared with those of *merA*::Tn5 mutants by plating on kanamycin-containing agar. The data in Table 4 show that *merA* mutations carried by pBR322-derived recombinant *mer* plasmids (pDU1003 and pDU962 *merA* mutants) conferred a greater level of hypersensitivity to Hg^{2+} (inhibitory zones of 23 to 26 mm) compared with other plasmids (inhibitory zones of 20 mm). The higher copy number of pBR322 might be responsible for the increased hypersensitivity phenotype. However, pDU962 and pDU1003 are both derived from pBR322, yet pDU1003 *merA* was more sensitive to Hg^{2+} than was pDU962 *merA*. Copy number may not be the only determinant of the level of *mer* protein synthesis.

Other experiments (see below) showed that complementation analysis could not be performed with multicopy pDU1003 *merA*::Tn5 mutants owing to the dominance of the multicopy hypersensitivity phenotype. This phenomenon was investigated with *merA* mutant plasmids of various copy numbers in cells carrying either pDU202 *mer+* or pDU3321 *merR* along with the multicopy *merA* mutant. In both cases the expected phenotype of the heterozygous cell was Hg^r owing either to the presence of the wild-type *mer* operon on pDU202 or because of complementation between pDU3321 *merR* *merA+* and the *merR+* *merA* element. The pBR322-derived *merA* mutants failed to show expression of Hg^r in these tests. Furthermore, the pDU1003 *merA* mutant exerted a greater dominance effect over the *mer+* and *merR* elements than pDU962 *merA* did. This correlates with the differences in hypersensitivity (Table 4) noted above and suggests that both effects may have a common cause.

Properties of pDU1124 and pDU1135, *mer*::Mu d *amp lac* derivatives of R100-1. Phage Mu d *amp lac* was inserted in the *mer* genes of plasmid R100-1 to generate *mer-lac* fusions which could be used to study regulation of *mer* gene expression. Plasmid pDU1124 is a derivative of R100-1 having Mu d *amp lac* inserted in the *merA* gene. The plasmid expressed an Hg^{2+} hypersensitive phenotype and complemented pDU3321 (*merR*) in phenocopy mating complementation tests, showing that its *merT* and *merR* genes were intact. Very low levels of β-galactosidase were synthesized by uninduced cultures (Table 5). These were increased to 374 U after induction with 0.5 μM Hg^{2+}. This is consistent with the observation that strains carrying this plasmid formed red colonies on MacConkey lactose agar only when subtoxic concentrations of Hg^{2+} were included and suggests that β-galactosidase is expressed from the *mer* operon promoter.

The *merR* gene of plasmid pDU1124 was inactivated by transferring the *merR*::Tn5 insertion mutation from plasmid pDU1145 (pDU991 *merR68*::Tn5) by homologous recombination as described above. The resulting strain pDU1161 *merR*::Tn5 *merA*::Mu d *amp lac* homogenote expressed about 45 U of β-galactosidase constitutively (Table 5). This presumably corresponds to the microconstitutive expression of mercuric reductase by R100-1 *merR* mutants (14).

The *merR-lac* fusion derivative of plasmid R100 (pDU1135) conferred an Hg^{2+} sensitive phenotype. It failed to complement pDU3321 (*merR*) but did undergo complementation with pDU3316 (*merA*), showing that its *merA* and *merT* genes were intact. This mutant expressed 300 U of β-galactosidase constitutively (Table 5), and it gave colonies growing on MacConkey lactose agar a red color. It is assumed that β-galactosidase is expressed from the *mer* promoter.

Characterization of Tn5 insertions in the *mer* genes of pDU1003 and pDU991. To obtain accu-

TABLE 4. Recombinant *mer* plasmids with *merA* mutations

Plasmid	Derivation	Sensitivity to HgCl₂: zone diameter (mm)[a]			Phenotype
		Alone	With pDU3321	With pDU202	
pDU1186	pDU202 *merA*::Tn801 tagged with Tn5	20			
pDU1187	pDU991 (ΔmerA) tagged with Tn5	20	0	0	Hypersensitive recessive
pDU1188	pDU989 *merA*::Tn5	20	0	0	Hypersensitive recessive
pDU1189	pRR130 *merA*::Tn5	20	4	3	Hypersensitive recessive
pDU1190	pDU962 *merA*::Tn5	23	7	9	Hypersensitive dominant
pDU1081	pDU1003 *merA53*::Tn5	26	15	16	Hypersensitive dominant

[a] Zone diameters were determined from mean values of two independent experiments. Cultures were flooded on λ base agar containing kanamycin (for hypersensitive mutant plasmids alone) or on λ base agar plus kanamycin and chloramphenicol for heterozygous strains. The diameter of the disk was 6 mm.

TABLE 5. β-Galactosidase activity expressed by mer-lac fusion plasmids[a]

Resident plasmid	Condition	β-Galactosidase activity (U) with mer-lac fusion plasmid:		
		pDU1124 (merA::Mu d amp lac)	pDU1161 (merR25::Tn5 merA::Mu d amp lac)	pDU1135 (merR::Mu d amp lac)
Alone	Uninduced	5	44	312
	Induced	374	45	327
With pDU1059 merA31::Tn5	Uninduced		8	43
	Induced		752	44
With pDU1053 merR25::Tn5	Uninduced		68	415
	Induced		64	415

[a] Host strain was ED2030 recA. Induction was with 0.5 μM HgCl₂. The mer-lac fusion plasmids pDU1124, pDU1161, and pDU1135 are derivatives of R100-1 (low copy number). pDU1059 and pDU1053 are derivatives of pDU1003 (high copy number).

rate physical coordinates of mer genes, a series of independent Tn5 insertion mutations in the mer region of the multicopy plasmid pDU1003 were isolated. The insertions were mapped as described above, and the Hg^{2+} sensitivity phenotype that they conferred was measured by disk tests. In the previous study of R100-1 mer mutants, two phenotypic types of mutant were defined, those conferring the same level of Hg^{2+} sensitivity as plasmid-free strains and those conferring Hg^{2+} hypersensitivity. The mer::Tn5 mutations in pDU1003 conferred a variety of Hg^s phenotypes with zones of inhibition ranging

from 8 to 24 mm beyond the 6-mm disk diameter (Table 6).

A cluster of mutations bounded by plasmids pDU1059 and pDU1068 (Fig. 2) conferred extreme Hg^{2+} hypersensitivity (zone of inhibition, 24 mm). These mutations spanned the EcoRI site known to be located in the merA gene (26) and did not allow synthesis of detectable mercuric reductase (data not shown). They were thus thought to define the merA gene, the structural gene for the mercuric reductase subunit.

From previous studies with plasmid R100-1 mutants, it was predicted that mutations in merR

TABLE 6. Sensitivity of HgCl₂ conferred by mer::Tn5 mutants and mer deletion mutants

Mutation	Sensitivity of HgCl₂ conferred by plasmid:								
	pDU1003		R100-1 merA⁺ mer::Tn5 homogenote		R100-1 merA::Mu d amp lac mer::Tn5 homogenote		pUB5572 mer deletion[a]		
	Plasmid	Inhibition zone (mm)[b]	Plasmid	Inhibition zone (mm)	Plasmid	Inhibition zone (mm)	Plasmid	Deletion	Inhibition zone (mm)[b]
merR25::Tn5	pDU1053	8		ND[c]		8			
merT61::Tn5	pDU1088	16		ND	pDU1163	5	pDU1180	Δ[merTCAD]61	11
merT44::Tn5	pDU1072	11	pDU1171	5	pDU1162	6	pDU1179	Δ[merTCAD]44	10
merC3::Tn5	pDU1033	19	pDU1174	14	pDU1166	15	pDU1181	Δ[merCAD]3	19
merC9::Tn5	pDU1039	19	pDU1175	13	pDU1167	15	pDU1182	Δ[merCAD]9	19
merA31::Tn5	pDU1059	24		ND		ND			ND
merD19::Tn5	pDU1047	12		ND		ND			ND
merR::Tn801	Control	ND	pDU3321	10		ND			ND
merA::Tn801 Control	Control	ND	pDU3316	15		ND			
Wild-type	pDU1003 mer⁺	6	R100-1 mer⁺	0	pDU1124 merA::Mu d amp lac	15	pDU989 pDU991	mer⁺ Δ[merAD]	4 20
No plasmid		10		10		10	R⁻		10

[a] The pUB5572 derivative plasmids were tested for Hg^{2+} sensitivity on M9 salts-Casamino acids agar containing trimethoprim. The mer⁺ plasmid pDU989 showed a small zone of inhibition on this medium. On LB agar it was fully resistant.

[b] Zones of inhibition minus the disk diameter (6 mm) with 10 μg of HgCl₂. Each number is the mean of two experiments.

[c] ND, Not determined.

and $merT$ would express Hg^{2+} sensitivity. The majority of Hg^s pDU1003 elements had insertions mapping proximal to $merA$, where $merR$ and $merT$ are known to be located (14). Unexpectedly, four insertions which conferred Hg^{2+} sensitivity (zones of inhibition, 11 to 12 mm; Table 6) mapped distal to the hypersensitive mutants. Furthermore, they expressed inducible reductase activity which was indistinguishable from that specified by the parental plasmid (Fig. 4). These mutations possibly define a new mer gene which we have tentatively called $merD$. The cell-free enzyme from these $merD$ mutants showed the same K_m and identical heat stability to wild-type R100 enzyme (F. D. Porter, unpublished results), making it rather unlikely that they have alterations in the 3' end of the $merA$ gene.

The insertions which mapped proximal to $merA$ were subdivided into three groups on the basis of Hg^{2+} sensitivity and map position. One group, located between the promoter-proximal $Hinc$II site and $merA$ (bounded by plasmids pDU1072 and pDU1079; Fig. 2), was generally more resistant to Hg^{2+} (zone of inhibition, 11 to 16 mm) than the promoter-distal cluster (bounded by pDU1033 and pDU1048; zone of inhibition, 19 mm; Fig. 2 and Table 6). The least sensitive mutant (zone of inhibition, 8 mm) mapped between the promoter-proximal $Hinc$II site and IS1. All these mutants expressed reductase constitutively at levels which varied from 0.5 to 10% of the induced control plasmid activity in broken cell extracts. This indicates that the
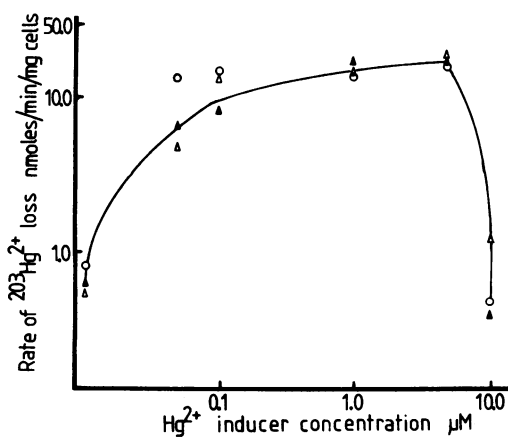

FIG. 4. Inducible volatilization activity of strains carrying pDU1003 $merD$::Tn5 mutant plasmids. Volatilization assays with 5 μM $^{203}Hg^{2+}$ were performed as described in the text after cultures were induced for 60 min with various concentrations of Hg^{2+}. The strains tested harbored plasmids pDU1047 $merD19$::Tn5 ($\triangle$), pDU1073 $merD45$::Tn5 ($\blacktriangle$), and pDU1003 mer^+ ($\bigcirc$).

$merA$ gene is intact but that regulation of its expression has been affected. Microconstitutive expression of reductase was reported previously for $merR$ Hg^{2+}-sensitive mutants mapping in the corresponding region of R100 (14).

Characterization of additional mutations affecting $merR$. Preliminary studies with plasmid pDU1003 mer::Tn5 insertions indicated that only one mutant (pDU1053) was defective in $merR$. To obtain more mutations for mapping experiments, Tn5 was inserted in the low-copy-number, hypersensitivity-conferring plasmid pDU991. Insertions either in $merR$ or $merT$ were expected to confer higher Hg^{2+} resistance owing to their failure to express transport function. They were distinguished by complementation tests with pDU3321 $merR$. Four $merR$::Tn5 mutations were isolated and mapped. Three were located between IS1 and the promoter-proximal $Hinc$II site; one (pDU1140) mapped within or near this $Hinc$II site.

To confirm that $merR$ extends to the $Hinc$II site, the DNA located between the $Hinc$II sites in the mer region of plasmid pDU1003 was removed in vitro. The resulting plasmid (pDU995; Fig. 2) conferred an Hg^{2+}-sensitive phenotype and failed to express $merR$ activity as monitored by the absence of induction of β-galactosidase activity in cells with R100-1 $merR$::Tn5 $merA$::Mu d lac amp (data not shown). Thus, pDU995 is defective in $merR$.

Characterization of a deletion mutation in the $merD$ region. To map the extent of the $merD$ gene (presumably identified by Tn5 insertion mutations in plasmid pDU1003), a deletion was generated which removed the 3,100-bp mer operon-distal $Hinc$II fragment of plasmid pDU1003 (giving pDU1214; Fig. 2). This element expressed the same multicopy Hg^{2+} resistance phenotype as pDU1003 (data not shown). Furthermore, when the copy number of this variant was reduced by cloning the PstI-BamHI fragment of pDU1214 into PstI-BglII-cleaved pUB5572 (to yield pDU1215), the Hg^r phenotype was indistinguishable from the wild type. Thus, $merD$ cannot extend past the $Hinc$II site distal to $merA$.

The doubt about $merD$ was increased when the copy numbers of the Hg^s plasmids pDU1003 $merD19$::Tn5 and $merD45$::Tn5 were reduced by homologous recombination with R100-1 and by cloning into pUB5572 to give variants which expressed wild-type Hg^r phenotypes (data not shown).

Characterization of $merA$::Mu d amp lac recombinants carrying mer::Tn5 mutations. The mer::Tn5 isolated in the multicopy plasmid pDU1003 could not be studied genetically in complementation tests with the earlier Tn801 mutations (14) because many of the multicopy

mutants were dominant to low-copy plasmid R100-1 variants, as described above. Mutations in *merR* and *merD* were recessive to pDU202 *mer*[+] but failed to perform as expected in complementation tests with pDU3316 and pDU3321, respectively (data not shown). Mutants defective in *merT* were dominant to pDU202 *mer*[+] and failed to complement pDU3321 (data not shown).

Because dominance interfered with genetic complementation, the copy numbers of several of the *mer*::Tn5 mutations were reduced by transferring the mutation by homologous recombination with R100-1 *merA*::Mu d *amp lac* (Fig. 3). In addition, several *merR*::Tn5 mutations in pDU991 were transferred by homogenotization into this R100-1 variant. Complementation tests were performed by phenocopy mating tests with pDU3316 and pDU3321. Mutations in *merR* complemented pDU3316 (*merA*) but not pDU3321 (*merR*), whereas mutants with a lesion elsewhere in the *mer* operon complemented pDU3321 (*merR*) but not pDU3316 (*merA*). It was useful to be able to compare the Hg phenotype and reductase expression by cells harboring the same mutation at high and low copy numbers and to be able to compare their phenotypes to known R100-1 *mer* mutants directly. Constitutive expression of reductase and β-galactosidase in the Lac and Lac[+] homogenotes was compared.

Disk sensitivity tests with the R100-1 *mer*::Tn5 homogenotes confirmed the suggestion that *merA*-proximal Hg[s] mutants were of two phenotypic types. Thus, the cluster (*merC*) closest to *merA* expressed a hypersensitive phenotype very similar to the control *merA* plasmid (Table 6). It is noteworthy that the *merA*[+] homogenotes were slightly less sensitive than the *merA*::Mu d *amp lac* variants, possibly because of microconstitutive reductase activity expressed by the former. The fact that reductase was produced by these insertion mutants indicates that they probably define a new *mer* gene, which we have called *merC*.

Binding of [203]**Hg**[2+] **promoted by mutant *mer* plasmids.** We previously reported a correlation between expression of Hg^{2+} hypersensitivity and inducible hyperbinding of [203]Hg^{2+}; this was attributed to the expression of the *merT*-encoded Hg^{2+}-specific transport function in the absence of reductase (14, 26). The genetic studies described here show that mutants defective in a region (*merC*) which is located promoter proximal to *merA* express hypersensitivity but also express low constitutive levels of reductase. To show that these mutations expressed inducible hyperbinding of Hg^{2+}, it was first necessary to generate deletions which inactivated *merA* since constitutive expression of reduc-

tase would have interfered with binding experiments. Deletions were generated by cloning the *Pst*I fragment carrying *mer* DNA proximal to the Tn5 insertion into plasmid pUB5572. Disk sensitivity tests showed that these deletion variants expressed Hg phenotypes similar to those of their parental plasmids (Table 6).

The data presented in Fig. 5 show that *merC* mutant plasmids pDU1181 and pDU1185 express inducible [203]Hg^{2+} hyperbinding activity indistinguishable from that of the pDU991 (Δ*merA*) control. In contrast, pDU1179 and pDU1180 (Δ*merTCAD*) lack hyperbinding activity. This confirms the identification of the *merT* gene and shows that *merC* plays no role in transport of Hg^{2+} as measured in the binding experiments.

Use of *mer-lac* fusions to study regulation of *mer* gene expression. The R100-1 *merR*::Tn5 *merA*::Mu d *amp lac* plasmid pDU1161 was used to test for the expression of *merR* in cells
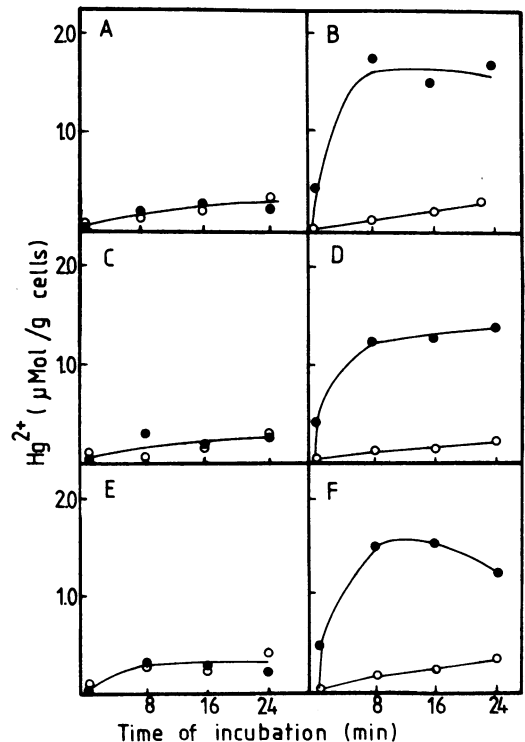

FIG. 5. Inducible hyperbinding activity of *mer* deletion mutants. Cultures carrying deletions generated by cloning *mer* sequences in plasmid pUB5572 were either uninduced (○) or induced (●) with 0.25 μM Hg^{2+} before being tested for hyperbinding of 5 μM [203]Hg^{2+} as described in the text. The plasmids tested were: (A) pUB5572, control without *mer*; (B) pDU991 Δ(*merAD*); (C) pDU1179 Δ(*merTCAD*)44; (D) pDU1181 Δ(*merCAD*)3; (E) pDU1180 Δ(*merTCAD*)61; and (F) pDU1185 Δ(*merCAD*)20.

carrying a multicopy *merA* mutant plasmid, which exerts a dominant hypersensitive phenotype in complementation tests. If *merR*$^+$ is supplied in trans, it should influence the microconstitutive expression of β-galactosidase from the *mer* operon promoter of pDU1140. A *merA*::Tn5 (pDU1059) derivative and a *merR*::Tn5 (pDU1053) derivative of pDU1003 were introduced into a *recA* strain carrying pDU1161. The level of expression of β-galactosidase was reduced to close to that of the *merR*$^+$ *merA-lac* parental plasmid by the *merR*$^+$ derivative but not by the *merR* derivative (Table 5). The presence of *merR*$^+$ also allowed the induction of β-galactosidase to a level similar to the R100-1 *merR*$^+$ *merA-lac* plasmid. Similar data were obtained when other *merR*::Tn5 homogenotes of pDU1124 were tested (data not shown). Thus, *merR* function supplied in *trans* appears to act both as an inducer and as a repressor of the *mer* operon. This was confirmed by performing analogous complementation experiments with pDU3321 (R100-1 *merR*::Tn801) and pDU1003 *merA*::Tn5 or *merR*::Tn5 elements and measuring the expression of mercuric reductase (data not shown).

To test the possibility that *merR* might be autoregulatory, the expression of β-galactosidase by strains harboring the *merR*::Mu d *amp lac* fusion plasmid pDU1135 was measured along with derivatives of pDU1003 (Table 5). A plasmid with an intact *merR*$^+$ gene (pDU1059 *merA*) repressed β-galactosidase activity by a factor of 8 to 10. This reduced expression could not be increased by preincubation with Hg^{2+}. Similar results were obtained with pDU1003 *mer*$^+$, *merC*, and *merT* plasmids. This repression was shown to be a consequence of *merR* gene expression because the *merR*::Tn5 mutant plasmid pDU1053 failed to reduce β-galactosidase activity (Table 5). These data suggest that

merR product negatively regulates *merR* gene expression.

The synthesis of mercuric reductase by *merR*, *merT*, and *merC* mutants located on the multicopy plasmid pDU1003 was compared with the level expressed by the corresponding R100-1 strains (Table 7). Each mutant specified low levels of reductase constitutively as estimated by direct measurement of enzyme activity in broken cell extracts (Table 7). The *merT*::Tn5 mutants expressed from 0.08 to 3% of the induced pDU1003 reductase levels and from 1 to 8% of the induced R100-1 reductase levels. The *merC* mutants yielded 0.3 to 2% of the induced pDU1003 levels, and the homogenotes yielded 0.1 to 11% of the R100-1 induced levels. It seemed that the expression of reductase was slightly higher for the R100-1 derivatives than for the pDU1003 mutants (when expressed as a percentage of the induced wild-type control level). Microconstitutive expression of Tn5-distal genes by *merR*, *merT*, and *merC* mutants was also shown by the levels of β-galactosidase in the *mer*::Tn5 *merA-lac* fusion strains (Table 7). The reduction in reductase and β-galactosidase synthesis in strains with proximal Tn5 insertions in the *mer* operon is due to polarity (1), probably caused by translational termination signals close to the outer ends of the inverted repeats of the Tn5 (H. Schaller, personal communication). The low constitutive expression is presumably due to promoter activity emanating from within Tn5 (2, 31) or from promotor sequences created by fusion of Tn5 to *mer* sequences, as has been suggested for *lac*::Tn5 insertions (1). Unfortunately, it was not feasible to determine the orientation of Tn5 by physical methods.

DISCUSSION

Previous genetic studies of the *mer* region of plasmid R100 identified two structural genes,

TABLE 7. Expression of mercuric reductase and β-galactosidase

| Mutation | Cell-free mercuric reductase | | | | β-Galactosidase (R100-1 *mer*::Tn5 *merA*::Mu d *amp lac*) | |
| | pDU1003 *mer*::Tn5 | | R100-1 *mer*::Tn5 | | | |
	Plasmid	Activity[a]	Plasmid	Activity	Plasmid	Activity
merR25::Tn5	pDU1053	0.5		ND[b]	pDU1161	4.2
merT44::Tn5	pDU1072	2.9	pDU1171	8.1	pDU1162	10.6
merT61::Tn5	pDU1088	0.08		ND	pDU1163	0.8
merT6::Tn5	pDU1036	0.7	pDU1172	1.2	pDU1164	7.9
merT51::Tn5	pDU1079	0.5	pDU1173	2.1	pDU1165	8.0
merC3::Tn5	pDU1033	1.5	pDU1174	3.9	pDU1166	8.6
merC9::Tn5	pDU1039	0.4	pDU1175	0.1	pDU1167	9.1
merC36::Tn5	pDU1064	0.3	pDU1176	0.1	pDU1168	8.4
merC24::Tn5	pDU1052	2.0	pDU1177	10.8	pDU1169	10.3
merC20::Tn5	pDU1048	1.5	pDU1178	4.6	pDU1170	10.2

[a] Expressed as percentage of activity in induced control strain.
[b] ND, Not done.

merT and *merA*, which were required for the expression of resistance to mercuric ions (14). The *merT* gene determines an Hg^{2+}-specific transport function, and *merA* specifies the mercuric reductase enzyme. These genes are thought to be coordinately expressed in an operon under the positive (and possibly negative) control of the product of another gene, *merR*. Evidence for the operon model came from polar effects of *merOP* and *merT* insertion mutants on the expression of the *merA* reductase gene and from complementation studies (14). In this paper, we report a more detailed analysis of the R100 *mer* genes which confirms the original model and gene order. Furthermore, we have tentatively identified two new *mer* genes, called *merC* and *merD*. The coordinates of the *mer* genes were deduced by mapping different *mer*::Tn5 insertions. This also allowed the minimum size of the genes to be estimated.

Mutations in *merR* occurred to the left and within the *Hinc*II site located close to IS*1* (Fig. 2). The mutations span a region of 370 bp, which could encode a polypeptide of about 13,500 daltons. Plasmids with *merR* mutations do not confer resistance to Hg^{2+} but do allow microconstitutive expression of *mer* operon genes as measured by mercuric reductase activity or β-galactosidase activity of a *merR*::Tn5 *merA*⁺ or *merA-lac* fusion. Studies with a *merR-lac* fusion suggest that the *merR* gene may be subject to negative autoregulation. However, the reduced expression of β-galactosidase in the presence of multicopy *merR*⁺ plasmids may be the result of overproduction of the regulatory protein.

Plasmid R100 *merR merA-lac* fusion derivatives and *merR merA*⁺ plasmids could be complemented by *merR*⁺ plasmids to express the same level of β-galactosidase or mercuric reductase as the corresponding *merR*⁺ strains. Repression in *trans* was also observed. Thus, the *merR* gene product can act as an inducer and repressor of the *mer* operon, as previously suggested (14).

A 260-bp *Eco*RI* fragment carrying a promoter subject to Hg^{2+}-inducible expression in the presence of a *merR*⁺ plasmid (3) also carried the *Hinc*II site, which we have now shown to be within *merR*. Since the *merR*::Tn5 insertion (pDU1140) located closest to *merT* can be complemented by *merR*⁺ elements, the operator region must be intact. Thus, the operator region for the *mer* operon lies to the right of the *Hinc*II site between *merR68*::Tn5 (pDU1140) and *merT44*::Tn5 (pDU1072).

The *merT* gene has been assigned the property of encoding an Hg^{2+}-specific transport function required for expression of resistance. The minimum size of *merT* is 340 bp, which could specify a polypeptide of 12,500 daltons. The characteris-

tic properties of *merT*::Tn5 mutations were that (i) they conferred sensitivity to Hg^{2+}, (ii) they expressed *merR*⁺ in complementation tests; (iii) they were *merA*⁺, although reductase activity was expressed constitutively at a low level owing to Tn5 polarity; and (iv) they did not show inducible hyperbinding of $^{203}Hg^{2+}$.

The earlier model for the genetic structure of the *mer* region (14) placed *merT* directly adjacent to *merA*. We have tentatively identified an additional gene (*merC*) which maps between *merT* and *merA*. The *merC*::Tn5 mutations had the following properties: (i) they expressed *merR*⁺ in complementation tests; (ii) they expressed *merT*⁺ as determined by their hypersensitive phenotype and inducible hyperbinding of $^{203}Hg^{2+}$; (iii) they were *merA*⁺ but expressed low constitutive levels due to Tn5 polarity; and (iv) they conferred levels of Hg^{2+} sensitivity intermediate between the sensitive *merT* plasmids and the hypersensitive *merA* elements. This intermediate sensitivity was probably due to the microconstitutive expression of mercuric reductase because *merC*::Tn5 ΔmerA double mutants conferred the same hypersensitivity phenotype as *merA* elements. Our studies have not revealed a function for *merC*, so it could be argued that the region between *merT* and *merA* is not a gene but a long untranslated intercistronic region and that the mutant phenotype of Tn5 insertions in this region is solely due to Tn5 polarity. However, the identification by DNA sequencing of an open reading frame in the corresponding region of the closely related Tn*501* element (N. L. Brown, R. D. Pridmore, and D. C. Fritzinger, personal communication) argues against this possibility.

Unexpectedly, four Tn5 insertions promoter distal to *merA* in plasmid pDU1003 conferred Hg^{2+} sensitivity. These strains synthesized mercuric reductase activity during a 60-min induction period at a rate indistinguishable from that of the wild-type control strain. The properties of their reductase (K_m and heat inactivation temperature) were identical to those of the enzyme from wild-type strains (F. D. Porter, personal communication), making it unlikely that these mutations affect the carboxy terminus of the reductase enzyme without affecting its ability to reduce Hg^{2+}. DNA sequencing of Tn*501* (N. L. Brown, R. D. Pridmore, and D. C. Fritzinger, personal communication) shows an open reading frame promoter distal to *merA*, which encourages us to believe that these insertions mark a new gene, which we have called *merD*. It is not possible to estimate the size of *merD* from these studies because only four closely linked insertions were mapped. However, the open reading frame in Tn*501* that corresponds to *merD* is 1.58 kb in length. A sequence of R100 DNA (T. K.

Misra and S. Silver, unpublished results) that starts with the HincII site (Fig. 2) and moves rightward shows close sequence homology to a sequence in the middle of the Tn501 reading frame, suggesting that the merD gene may extend beyond the HincII site. However, the deletion of the HincII fragment in pDU1214 (Fig. 2) did not affect the Hg^r phenotype of either high-copy-number or low-copy-number plasmids (data not shown). This result argues against the merD gene extending beyond the HincII site.

Transposon Tn5 has been reported to insert virtually at random within short stretches of DNA but shows a regional specificity for AT-rich areas (24, 27). The distribution of Tn5 insertions among the mer genes of pDU1003 (5 in merR, 4 in merT, 13 in merC, 30 in merA, and 4 in merD) might reflect the relative sizes of the genes as well as the phenotype conferred by the mutation. The phenotype of pDU1003 merR is very similar to that of pDU1003 itself in plating tests (zones of inhibition of 8 and 6 mm, respectively). When a different method of identifying merR mutations was used, several more were obtained without difficulty. This might also explain the small number of merD insertions isolated.

Up to seven Hg^{2+}-inducible polypeptides have been observed in λmer-infected cells (12) and in minicells with plasmid pRR130 (18). We have observed five Hg^{2+}-inducible polypeptides encoded by plasmid pDU1003 in the minicell system (unpublished data). Preliminary experiments suggest that a polypeptide of 11,000 daltons is specified by merT and one of 13,750 daltons is specified by merC. No polypeptide has been associated with the merD region as yet.

The mer region of plasmid R100 is closely related to that of Tn501. Five open translational reading frames exist in the mer region of Tn501 and could correspond to merR, merT, merC, merA, and merD (N. L. Brown, R. D. Pridmore, and D. C. Fritzinger, personal communication). DNA sequencing results with pDU1003 have shown approximately 90% base identities in the merR, merOP, merT, merA, and merD regions of the elements (T. K. Misra and S. Silver, unpublished data). This suggests that the gene order in Tn501 is likely to be the same as that which we have suggested for R100.

The function of the putative merD gene in the expression of Hg^{2+} resistance also presents a problem. First, the merD::Tn5 mutations from multicopy plasmid pDU1003 did not confer Hg^{2+} sensitivity when the gene copy number was reduced. Thus, R100-1 merD::Tn5 plasmids and pUB5572 merRTCA$^+$ ΔmerD derivatives (generated by cloning) expressed wild-type Hg^r phenotypes. Second, removal of the 3,100-bp merA-distal HincII fragment (which removes a substantial part of the DNA homologous to the long merD reading frame in Tn501) did not alter the Hg^r phenotype of pDU1003. Furthermore, lowering the copy number of this deletion by cloning into pUB5572 yielded a variant which expressed the normal wild-type Hg^r phenotype. Thus, merD does not seem to contribute towards expression of Hg^r by low-copy-number wild-type mer plasmids in Escherichia coli. The pDU1003 merD::Tn5 mutations may be artifacts exerting an upstream polar effect.

The Hg^{2+} resistance level did not increase when the copy number of the plasmid R100 mer genes was increased by cloning into ColEl::TnA (25). Furthermore, no increase in whole cell volatilization activity was detected, despite the synthesis of elevated levels of reductase detectable in broken cells. This cryptic mercuric reductase activity was attributed to a limitation in the rate of merT-dependent transport of Hg^{2+} across the membrane (25). The expression of mer genes at different copy numbers was studied further here. Plasmids pDU1003 and pDU1125 (pBR322 mer$^+$ and pACYC184 mer$^+$; copy number, 20 to 30 per cell [4, 7]) produced more reductase than pRR130 (derived from ColEl; 16 copies per cell [5]) or R100 (1 to 2 copies per cell [28]) (Table 3). As was shown before, this activity was cryptic in whole cells. Furthermore, the plasmids which expressed the greatest cell-free mercuric reductase activity (pDU1003 and pDU1125) conferred lower levels of resistance than did pDU202, pDU989, and pRR130 (Table 3). This can be explained if the mer operon proteins, including the transport protein, are overproduced to the extent that resistance is not expressed properly. The cell may become flooded with Hg^{2+} faster than reductase can remove it. This hypothesis is supported by the finding that multicopy merA mutant plasmids conferred increased Hg^{2+} hypersensitivity, which suggests that the transport functions are overproduced, resulting in a gene dosage effect for Hg^{2+} transport rate.

Another manifestation of the imbalance between mercuric reductase activity and Hg^{2+} transport is the observation that merA mutations on multicopy plasmids pDU962 and pDU1003 were dominant and prevented the expression of wild-type Hg^r by a low-copy mer$^+$ plasmid in the same cell. The mer operon of the low-copy plasmid in these heterozygous cells was inducible because mercuric reductase activity increased after induction, as did β-galactosidase in merA-lac fusion strains.

R100-1 mer::Tn5 mutants with Tn5 insertions located promoter proximal to the merC gene showed Hg^{2+} sensitivity similar to pDU3221 merR::Tn801. They also produced microconstitutive levels of reductase or β-galactosidase.

Complementation tests showed that five insertions were located in *merR*, and four were in *merT*.

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