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Transposon *A*-Generated Mutations in the Mercuric Resistance Genes of Plasmid R100-1

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A series of 23 transposon *801*(Tn*801*)-induced mutations of plasmid R100-1 from mercuric salts resistance to sensitivity was studied. Although Tn*801* transposed frequently into the *mer* region of the plasmid, fine structural analysis showed that the site of insertion within *mer* varied. About one-half of the Tn*801* insertion events also caused a deletion of greater than 1 megadalton. Genetic and restriction endonuclease *Eco*RI and *Bam*HI analysis of the mutant plasmid deoxyribonucleic acid elucidated the organization of the *mer* operon and suggested the existence of a *trans*-acting regulatory factor governing resistance to mercuric salts. Tn*801* insertions leading to mercuric sensitivity occurred in the restriction endonuclease fragments *Eco*RI-H and *Eco*RI-I. Regulatory mutations leading to a 50-fold-reduced synthesis of mercuric reductase enzyme occurred in two complementation classes thought to represent the gene for a *trans*-acting inducer molecule and a *cis*-acting operator-promoter sequence. Mutations leading to total loss of the enzyme mercuric reductase occurred on both the *Eco*RI-H and *Eco*RI-I fragments, showing that the structural gene for this enzyme (*merA*) bridges the *Eco*RI cleavage site separating the segments. Hypersensitivity to mercuric salts resulted when Tn*801* insertion occurred in the reductase gene in the operator-distal portion of the operon. Hypersensitive cells inducibly bound three to five times more Hg^{2+} at low concentrations than did sensitive (plasmidless) cells. This finding led to the proposal that another gene (*merT*) controls uptake of Hg^{2+} by the cells. Transcription of the operon was deduced to start in the *Eco*RI-H fragment and to move into the *Eco*RI-I fragment of the plasmid genome.

Plasmid-determined mercury resistance in bacteria is due to the enzymatic transformation of Hg^{2+} to Hg^0, which is volatilized from the growth medium (38, 43, 44). The enzyme responsible is the flavoprotein mercuric reductase. It is an intracellular enzyme which requires NADPH as a cofactor and the synthesis of which is inducible by subinhibitory concentrations of mercuric ions (16, 22, 38, 39, 43). In addition, plasmids such as R100 (also known as NR1 or R222) confer resistance to the organomercurials merbromin and fluoresceinmercuric acetate (46). Resistance to these organomercurials is not due to enzymatic degradation and volatilization of mercury. Recent studies have shown that the mercuric-organomercurial resistance (*mer*) genes of R100 are located on restriction endonuclease *Eco*RI fragments H and I (27, 30) and that at least three *mer*-specific polypeptides are formed after induction (8).

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Transposons are short stretches of DNA, usually bounded by terminally repeated sequences, that have the capacity to transpose from one replicon to another by a *recA*-independent recombination process (4, 7, 23, 36). Transposons cause mutations when they insert into a structural gene (23) and are polar on promoter-distal genes in an operon (24). Thus, transposons can be used as mutagenic probes to study the structure and function of the recipient DNA molecule (3, 9, 15, 21, 36; N. Kleckner, K. Reichardt, and D. Botstein, Genetics, in press). The mutational site is marked by a selectable drug resistance phenotype which aids in subsequent genetic manipulations. Furthermore, the characteristic structure of the transposon allows physical mapping of the insertion site.

Transposon *A* (Tn*A*; ampicillin resistance) has been shown to insert almost at random in small plasmids, such as ColE1 and RSF1010 (19, 21). Preliminary studies with plasmid R100-1 showed that insertion of Tn*A* led to mercuric ion sensitivity at a relatively high frequency. We

therefore undertook a combined biochemical and genetic analysis of the *mer* region of this plasmid with Tn*A* as a mutagenic probe.

MATERIALS AND METHODS

Bacterial strains and plasmids. The parental strains and plasmids used in this study are listed in Table 1.

Culture media. The compositions of the nutrient broth and agar (13) and tryptone broth (39, 43) were described previously, as was the minimal medium used for plating (14). A supplemented M9 minimal medium was used to culture bacteria for plasmid DNA isolation.

Isolation of Tn*801* insertion mutants of pDU202. The R plasmids RP1 (which carries the Tn*A* element Tn*801*) and pDU202 (a tetracycline-sensitive deletion mutant of R100-1, which is a mutant of plasmid R100 derepressed for conjugal transfer [10]) were introduced one at a time by conjugation into the *recA* strain ED2030. This plasmid-diploid strain was mated for 2 h at 37°C with an equal volume of a culture of strain JC3272, which is Str^r. Dilutions were plated onto agar selective for Amp^r [Str^r] and Chl^r [Str^r] progeny in order to measure the transfer frequency of the plasmids and to select for transposition of Tn*801* onto plasmid pDU202.

Resistance level determinations. Dilutions of $HgCl_2$, merbromin, and fluoresceinmercuric acetate were incorporated into nutrient broth agar. Overnight cultures of the mutant and control strains were diluted by 10^4, and about 0.01 ml was inoculated onto the surface of each plate with a phage-typing apparatus. Up to 25 different cultures were inoculated onto each series of plates. After overnight incubation the highest concentration of mercurial allowing normal colonial growth was recorded as the resistance level (procedure used in Dublin). Alternatively, mercuric sensitivity and resistance were determined by liquid growth experiments and by measuring the zone of inhibition surrounding antibiotic testing disks containing mercuric ion or organomercurials (procedure used in St. Louis) (32).

Determination of the orientation of Tn*801*. The pDU202*mer*::Tn*801* mutants and the parental pDU202 plasmid were transferred by conjugation into strain UB281 and strain UB1731, a derivative of UB281 carrying Tn*A* in the chromosome between *his* and *trp*. These strains were grown to 2×10^8 cells per ml and mated with strain JC3272 for 60 min at 37°C with gentle shaking. Appropriate dilutions were plated onto medium selective for His$^+$ [Pro$^+$ Met$^+$ Str^r] and Trp$^+$ [Pro$^+$ Met$^+$ Str^r] recombinants.

Reversion tests. Five independent 1-ml broth cultures of each of the sensitive mutants were grown overnight, and 0.1 ml was spread onto agar containing 55 μM $HgCl_2$. A larger number of cells of the hypersensitive mutants could be plated onto agar before interference by a background lawn of growth occurred.

TABLE 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype/phenotype[a]	Characteristics	Reference
Strain			
JC3272	*his trp lys lac rpsL*		1
ED2030	*trp lac rpsL recA*		14
DU1040	*his lac rpsL nalA*		11
DU1041	*pro thi lac rpoB*		11
KP245	*met his trp thy lac gal tsx*		30
UB281	*pro met nalA*		4
UB1731	*pro met nalA bla*	Carries Tn*A* (Tn*802*) in the chromosome between *his* and *trp*	4
Plasmid			
RP1	Kan^r Tet^r Amp^r IncP	Amp^r marker is located on Tn*A* (Tn*801*)	18
R1	Kan^r Tet^r Chl^r Amp^r Str^r-Spc^r Sul^r IncFII		31
pDU202	Chl^r Str^r-Spc^r Sul^r Mer^r IncFII	Tet^s deletion mutant of R100-1	15
R828	Amp^r Chl^r Gen^r Kan^r Str^r Tet^r Mer^r	IncL	38
pTK4	Mer^s derivative of R828	Isolated after chemical mutagenesis in this study	
pRR132	ColE1::Tn*A*-*Eco*RI-I of NR1		30
pRR134	ColE1::Tn*A*-*Eco*RI-H of NR1		30
pDU3300-pDU3308, pDU3315-pDU3328	Mer^s Amp^r Chl^r Str^r-Spc^r Sul^r	pDU202*mer*::Tn*801* mutants isolated in this study	

[a] *E. coli* genotype designations follow the current standard nomenclature (2) for chromosomal genes. *bla*, Beta-lactamase synthesis due to Tn*A*. The plasmid resistance markers are abbreviated as follows: kanamycin, Kan^r; tetracycline, Tet^r; chloramphenicol, Chl^r; gentamicin, Gen^r; streptomycin, Str^r; spectinomycin, Spc^r; sulfonamides, Sul^r; ampicillin, Amp^r; and mercuric ions, Mer^r.

Thus, for these strains five independent 10-ml cultures were grown to saturation and concentrated 10-fold before 0.1 ml was spread onto Hg^{2+}-containing agar. Revertant colonies were also detected and isolated from the inhibition zones around the paper testing disks (see Fig. 1D).

Recombination and complementation tests. The pDU202 variants were tested for recombination and complementation by performing conjugation experiments between pairs of mutants. Crosses were done in both directions, using each mutant plasmid both as a donor and in a recipient strain. Either the Rec^+ host DU1040 or the $RecA^-$ strain ED2030 carrying the plasmids was grown overnight in nutrient broth with shaking. The strains to be used as recipients were kept in stationary phase (in order to preserve the reduced surface exclusion level of plasmid-containing stationary-phase cells) and were diluted 1:5 immediately before mating. The donors were diluted in fresh broth and incubated until they reached about 2×10^8 cells per ml. Equal volumes of donors and recipients were mixed, incubated for 90 min at 37°C, and spread onto agar containing 55 μM $HgCl_2$. The occurrence of complementation was indicated by confluent growth on the selective plates after overnight incubation. To detect recombination, it was often necessary to concentrate the mating mixtures 10-fold. This could only be achieved without interference by background growth in crosses between the hypersensitive mutants.

Similar matings were performed with strain DU1041 *pro thi recA*$^+$ carrying the pDU202 variants as donors and strains carrying the nonconjugative hybrid plasmids pRR132 and pRR134 as recipients. The recipient cultures were prepared by overnight growth on the surface of nutrient agar plates containing 500 μg of carbenicillin per ml. The cells were harvested from the plates into broth and used in matings at a density of about 2×10^8 cells per ml. Stable plasmid diploids carrying the pDU202*mer*::Tn*801* variants and pRR132 or pRR134 in strain KP245 were obtained by selection for Chl^r [Pro^+ Thi^+] recombinants. These diploid strains were purified on selective medium and grown overnight on the surface of nutrient agar plates containing 20 μg of chloramphenicol per ml. The cells were harvested into broth and adjusted to 10^9 cells per ml before being plated onto Hg^{2+}-containing agar to detect mercuric-resistant recombinants.

Purification of plasmid DNA. Covalently closed circular pDU202*mer*::Tn*801* DNA was isolated by the cleared-lysate dye-buoyant density gradient centrifugation technique (6).

Restriction endonuclease cleavage and agarose gel electrophoresis. The restriction enzymes *Eco*RI and *Bam*HI were obtained either from M. Kehoe or from Miles Laboratories, Stoke Poges, England. Cleavage reactions were performed by standard procedures (for *Eco*RI see references 19 and 45; for *Bam*HI see references 26 and 36).

Mercury volatilization. Mercury volatilization assays for mercuric reductase activity were carried out as described previously (38, 39).

Binding experiments. Binding experiments with $^{203}Hg^{2+}$ were carried out as described in an accompanying paper (32). *mer* operon induction for both volatilization and binding assays was accomplished (unless otherwise noted) by addition of a low, subtoxic concentration of Hg^{2+} (generally 1 or 0.25 μM for hypersensitive strains) to early log-phase cultures, followed 30 min later by supplementation to a higher Hg^{2+} concentration (generally 5 μM) and followed 60 min after that by harvesting of the induced cells.

RESULTS

Isolation and characterization of pDU202*mer*::Tn*801* mutants. The following procedure was devised to select for the transposition of Tn*801* from plasmid RP1 to pDU202. Strain ED2030 carrying RP1 and pDU202 was mated with recipient JC3272. RP1 was transferred at a low frequency (5×10^{-3} per donor cell), whereas pDU202 was transferred at a higher frequency (0.4). Transconjugant cells carrying RP1 were resistant to kanamycin and tetracycline; transconjugant cells carrying pDU202 were resistant to chloramphenicol. Cells that were resistant to chloramphenicol and ampicillin, but sensitive to kanamycin and tetracycline, were assumed to have arisen by the transfer of pDU202::Tn*801* recombinant plasmids. These occurred at frequencies of 2×10^{-3} among the chloramphenicol-resistant transconjugants and 0.5 to 0.8 among the ampicillin-resistant transconjugants. Of the cells with the pDU202::Tn*801* plasmids, 2.5% (16 of 618 in one experiment) were sensitive to mercuric ions, but less than 0.1% were defective in antibiotic resistance markers of pDU202 (Str^r-Spc^r, Chl^r, or Sul^r). Since chloramphenicol-resistant transconjugants from donor cells carrying only pDU202 were all mercuric resistant (442 of 442), these results suggest preferred insertion of Tn*801* into the *mer* region of plasmid pDU202. The putative Mer^s mutants were transferred by conjugation to strain DU1040 by selecting for Chl^r [Nal^r] progeny after 2-h matings. In each case the Amp^r phenotype was cotransferred with Chl^r. Furthermore, the Amp^r, Spc^r, Sul^r, and Chl^r markers were cotransducible with bacteriophage P1. These tests ensured that the Amp^r marker occurred on plasmid pDU202.

Phenotypic properties of the mutants. Strains carrying 1 of the 23 independent pDU202*mer*::Tn*801* mutants, as well as control plasmids, were tested quantitatively for sensitivity to $HgCl_2$, merbromin, and fluoresceinmercuric acetate. Two classes of mutants were distinguished, those which were as sensitive to the mercurials as the plasmidless host and those which were four- to sevenfold more sensitive (Table 2 and Fig. 1). Considerable care was required in growth experiments with hypersensitive mutants. Cultures of these mutant strains contained a subpopulation of normally sensitive cells at a level of 10^{-4} to 10^{-3} in the absence of

TABLE 2. *Mercury and organomercurial sensitivities of pDU202mer::Tn801 mutants*

Class/controls	Resistance level on plates[a]		
	Hg^{2+} (μM)	Merbromin (μM)	Fluorescein-mercuric acetate (μM)
Hypersensitive: pDU3302, pDU3303, pDU3304, pDU3315, pDU3316, pDU3317, pDU3319, pDU3320, pDU3322, pDU3323, pDU3325, pDU3326, and pDU3328	1.7 ± 0.2	4.2 ± 0.5	1.5
Sensitive: pDU3300, pDU3301, pDU3305, pDU3306, pDU3307, pDU3308, pDU3318, pDU3321, pDU3324, and pDU3327	11.1 ± 0.7	24 ± 2	6
Controls			
pDU202 (Mer^r)	74	53	18
R1 (Mer^s plasmid)	10	27	6
R^- host cell (Mer^s)	10	25	6

[a] Average resistance level for three independent experiments with each plasmid-containing strain (± standard deviation). No reproducible difference in sensitivity was found within each class of pDU202*mer*::Tn*801* mutants. The plasmid host cell and the plasmid-less control was strain DU1040.

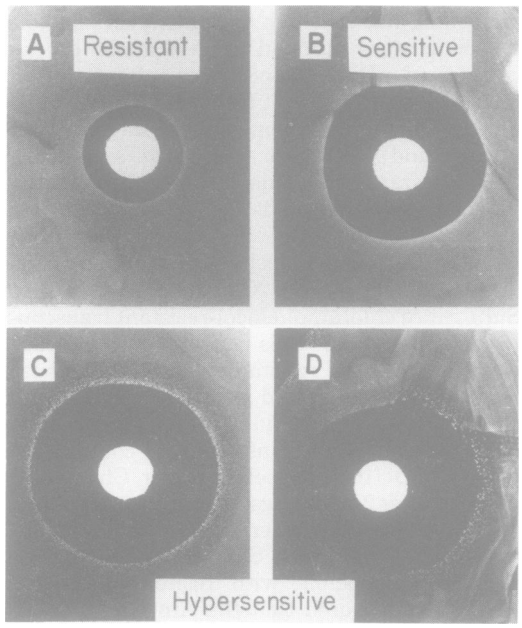

FIG. 1. *Zones of growth inhibition surrounding disks containing 100 nmol of Hg^{2+}. (A) Resistant strain DU1040(pDU202). (B) Sensitive R^- strain DU1040. (C) Hypersensitive strain DU1040-(pDU3302). (D) Hypersensitive strain DU1040-(pDU3303).*

Hg^{2+}. This subpopulation included cured cells that had lost the plasmid and cells carrying deletion mutants that had lost at least the proximal region of the *mer* operon. Analysis of these deletion mutants is described in an accompanying paper (12). The sensitive subpopulation eventually overgrew the hypersensitive strains in liquid growth experiments with Hg^{2+} and formed a "halo" of sensitive-cell microcolonies in the inhibition zones (Fig. 1C and D). Additional data showing the reproducibility of the resistance, sensitivity, and hypersensitivity levels are found in an accompanying paper (32).

Physiological properties of the mutants. Since mercuric resistance is due to the volatilization of metallic Hg^0 from the culture medium, the ability of each of the 23 pDU202*mer*::Tn*801* mutant plasmids to confer volatilization activity upon *Escherichia coli* cells was tested. In 18 of the 23 cases, no detectable enzyme activity was found, with a limit of resolution below 0.1% of the wild-type activity. Strains bearing the other five plasmids (pDU3300, pDU3305, pDU3307, pDU3321, and pDU3324) showed a low level of volatilization activity (Table 3). This activity differed from that of the wild type in that it was expressed constitutively in the absence of enzyme induction with low levels of Hg^{2+}. With the wild-type plasmid, the mercury volatilization activity was completely inducible, with less than 1/1,000th the maximum activity found with uninduced cells (Table 3). The naturally occurring R factor R1, which shares extensive DNA sequence homologies with R100 (40) but does not confer mercuric resistance, is known to have Tn*A* inserted in the region of the r-determinant that corresponds to *mer* in R100 (17, 33). Bacteria with plasmid R1 constitutively produced low levels of mercuric reductase activity, comparable to the levels produced by the five laboratory-generated strains (data not shown). We interpret this microconstitutive enzyme synthesis by cells with these six plasmids in terms of regulatory gene mutations. When non-volatilizing cells carrying mutant plasmids conferring

TABLE 3. *Microconstitutive mercuric reductase activity of some pDU202mer::Tn801 mutants*

Plasmid	Mercuric reductase activity (%)[a]	
	Uninduced	Induced
pDU3303 (hypersensitive)	≤0.04	≤0.04
pDU3303-1 (resistant)	0.05	100.
pDU3305 (sensitive)	2.0	1.7
pDU3307 (sensitive)	1.7	1.4
pDU3321 (sensitive)	1.7	1.2
pDU3324 (sensitive)	1.7	1.1

[a] Induced resistant rate normalized to 100% corresponded to 7.0 μmol/min per g of cells with 5 μM ^{203}Hg. The assays were carried out at 37°C in complete assay buffer (39) containing 200 μg of chloramphenicol per ml to prevent induction during the assay. The uninduced resistant rate resulted in loss of 6% of the radioactivity within 90 min; the half-life for loss by the induced resistant cells was 0.5 min, and with the microconstitutive mutants, the half-lives ranged from 25 to 45 min. In other experiments, plasmids pDU3300 and R1 were shown to have a quantitatively indistinguishable low level of microconstitutive reductase synthesis that was not increased upon induction.

sensitivity or hypersensitivity were ruptured by a French press and assayed for in vitro enzyme activity (39), none was found. When cells showing the low-level constitutive volatilizing activity were ruptured and assayed for in vitro volatilization, low levels were found, which was consistent with expectations from the whole-cell volatilization rates. No examples of slowly volatilizing cells giving rise to actively volatilizing cell-free preparations (i.e., cryptic mutants) were found.

Binding of radioactive $^{203}Hg^{2+}$ by sensitive and hypersensitive mutants. We hypothesized (31, 32) that hypersensitivity toward Hg^{2+} salts might be due to the presence of a plasmid-determined mercuric uptake system functioning in the absence of mercuric reductase. Therefore, we measured the uptake of radioactive mercury by sensitive and hypersensitive strains in the presence and absence of preinduction by low nontoxic levels of Hg^{2+}. Induced bacteria with plasmids conferring hypersensitivity to Hg^{2+} bound three to five times more $^{203}Hg^{2+}$ than did sensitive plasmidless or sensitive mutant plasmid cells (Fig. 2). The microconstitutive strains with plasmids pDU3307 and pDU3321 bound no more Hg^{2+} when induced than when not induced and volatilized one-half of the added 2 μM $^{203}Hg^{2+}$ from solution in 30 min. The conditions for Hg^{2+} binding by the sensitive and hypersensitive strains are analyzed in greater detail with the cloned *mer* operon fragments in an accompanying paper (32).

Reversion tests. Two of the pDU202*mer*::Tn*801* mutants (pDU3300 and pDU3303) reverted to mercurial resistance at a relatively high frequency (Table 4). Two others formed mercurial-resistant colonies at a lower level. However, 18 did not show revertants to mercurial resistance at detectable rates. Thus, only a small proportion of the putative *mer*::Tn*801* insertion mutants formed revertants. This suggests either that Tn*801* did not excise precisely at frequencies associated with other transposons

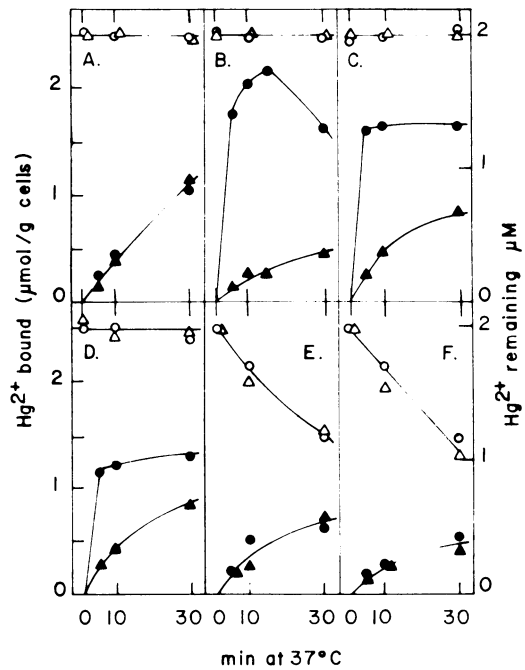

FIG. 2. *Binding of radioactive Hg^{2+} by sensitive and hypersensitive cells. (A) Strain DU1040 [cured variant of DU1040(pDU3325)]. (B) Strain DU1040(pDU3303), hypersensitive. (C) Strain DU1040(pDU3316), hypersensitive. (D) Strain DU1040(pDU3302), hypersensitive. (E) Strain DU1040(pDU3307, sensitive microconstitutive. (F) Strain DU1040(pDU3321), sensitive microconstitutive. Cells were grown in tryptone broth with or without induction by the addition of 0.2 μM Hg^{2+} at 0.22 g (dry weight) per liter. After harvesting at 0.34 g (dry weight) per liter, the volatilization of 2 μM $^{203}Hg^{2+}$ was measured by direct sampling or uptake of $^{203}Hg^{2+}$ by membrane filtration plus washing twice with 5 ml of broth. Symbols: ●, uptake by induced cells; ▲, uptake by uninduced cells; ○, remaining radioactivity with induced cells; △, remaining $^{203}Hg^{2+}$ with uninduced cells. The assays were with 0.68 g (dry weight) of cells per ml in tryptone broth containing added 2 μM $^{203}Hg^{2+}$, 0.1 mM Mg^{2+}, 0.1 mM EDTA, and 200 μg of rifampin per ml (to prevent induction during the assays).*

or that the insertion of Tn*801* irreversibly damaged the surrounding host sequences in some cases, perhaps by causing deletions during or after the transposition event.

Complementation tests. Most of the pDU202*mer*::Tn*801* mutants could be divided into two complementation groups (groups A and B) by their ability to form mercuric-resistant colonies at a high frequency in crosses with other mutants (Table 5). Since the crosses were between incompatible pDU202 derivatives, the cells selected on Hg^{2+}-containing agar rapidly segregated Hg^{2+}-sensitive cells during growth without selection. A third class (five mutants) did not complement mutants in either group A or group B, and these plasmids were subsequently shown to contain extensive deletions. To confirm that the mercuric-resistant progeny were formed by complementation and not recombination, the crosses were repeated in *recA* host cells with identical results. The number of heterozygous cells could not be estimated due to the lack of differential selective markers on the plasmids. Consequently, the frequency of formation of mercuric-resistant colonies per heterozygote could not be calculated.

The three mutants in complementation group A (pDU3307, pDU3321, and pDU3324) all formed mercuric-resistant colonies at a high frequency in crosses with strain KP245 harboring plasmid pRR134, which carries cloned NR1 *Eco*RI fragment H (Table 5). Similar results were obtained when stable pDU202*mer*::Tn*801*/pRR134 diploids (obtained without selection for mercuric resistance) were tested. Thus, the factor required to complement the group A pDU202 mutants was being provided by a *trans*-acting gene which is located on *Eco*RI fragment H. The wild-type plasmid R1 behaved in recombination and complementation experiments like a group B mutant (Table 5).

Mapping the pDU202*mer*::Tn*801* mutants by recombination tests. The ordering of many of the mutational sites within the *mer* region was achieved by mating pairs of noncomplementing mutants under conditions which allowed the detection of low levels of recombination and also by performing crosses with plasmids pRR132 and pRR134 (Table 5). These results are summarized in a deletion map (Fig. 3). The frequency of recombination between pairs of pDU202*mer*::Tn*801* mutants was very low (two orders of magnitude lower than in crosses with pRR132 or pRR134), even between pairs of mutants that were shown by other techniques to have quite widely separated lesions. This may be caused by the large Tn*801* sequence present on each molecule which interferes with recombination between homologous stretches of R100 DNA.

TABLE 4. *Frequencies of reversion to mercuric resistance*

Mutant plasmid	Reversion frequency[a]
Sensitive class	
pDU3300	1×10^{-6}
pDU3305	1×10^{-8}
pDU3301, pDU3306, pDU3307, pDU3308, pDU3318, pDU3321, pDU3324, and pDU3327	Nonreverting, $\leq 5 \times 10^{-9}$
Hypersensitive class	
pDU3302	1×10^{-9}
pDU3303	3.8×10^{-7}
pDU3316	5×10^{-10}
pDU3304, pDU3315, pDU3317, pDU3319, pDU3320, pDU3322, pDU3323, pDU3325, pDU3326 and pDU3328	Nonreverting, $\leq 5 \times 10^{-10}$

[a] Conditions, as described in the text, allowed greater resolution with hypersensitive strains than with mercury-sensitive derivatives.

It was possible to assign many of the Tn*801* insertions to either cloned *Eco*RI fragment H (pRR134) or cloned *Eco*RI fragment I (pRR132) by showing that mercuric-resistant recombinants occurred with only one of the hybrid plasmids (Table 5). Three of the hypersensitive mutants (pDU3315, pDU3325, and pDU3328) recombined with plasmid pRR132, whereas eight additional mutants (both sensitive and hypersensitive classes) recombined with plasmid pRR134. The occurrence of hypersensitive mutants (i.e., lacking the reductase volatilization activity but retaining hyperbinding activity [see above]) that can recombine with both *Eco*RI fragments shows that the reductase gene straddles the *Eco*RI site, separating the H and I fragments (Fig. 3). Seven mutant plasmids listed in Table 5 failed to yield recombinants with either cloned *Eco*RI fragment, because they carried deletions covering parts of both *Eco*RI fragments (see DNA analysis below). These included hypersensitive mutants (pDU3304, pDU3317, pDU3322, and pDU3323) and sensitive mutants (pDU3301, pDU3306, and pDU3318) (Fig. 3). Two additional deletion mutants (pDU3308 and pDU3327) were unable to complement either group A or group B mutants, but did form recombinants with pRR134. All of these deletion mutants failed to recombine with pDU202*mer*::Tn*801* point insertion mutants (Fig. 3).

Five mutational sites were identified in the reductase gene by recombination tests, although

TABLE 5. *Recombination and complementation between the pDU202mer::Tn801 mutants and pRR132 or pRR134*

pDU202 mutant	Complementation group	Sensitivity	Frequency of recombination with:[a]	
			pRR132	pRR134
pDU3307	A	Sensitive	0	1×10^{-2}[b]
pDU3321	A	Sensitive	0	1×10^{-2}[b]
pDU3324	A	Sensitive	0	1×10^{-2}[b]
pDU3300	B	Sensitive	NT[c]	NT
pDU3305	B	Sensitive	0	3×10^{-5}
R1	B	Sensitive	0	1×10^{-4}
pDU3302	B	Hypersensitive	0	1.4×10^{-6}
pDU3316	B	Hypersensitive	0	2.6×10^{-5}
pDU3319	B	Hypersensitive	0	1.6×10^{-5}
pDU3320	B	Hypersensitive	0	1×10^{-6}
pDU3326	B	Hypersensitive	0	5×10^{-5}
pDU3304	B	Hypersensitive	0	0
pDU3317	B	Hypersensitive	0	0
pDU3322	B	Hypersensitive	0	0
pDU3323	B	Hypersensitive	0	0
pDU3315	B	Hypersensitive	7×10^{-4}	0
pDU3325	B	Hypersensitive	7.4×10^{-6}	0
pDU3328	B	Hypersensitive	2.6×10^{-5}	0
pDU3303	B	Hypersensitive	NT	NT
pDU3308	Noncomplementing	Sensitive	0	1.2×10^{-6}
pDU3327	Noncomplementing	Sensitive	0	1×10^{-4}
pDU3301	Noncomplementing	Sensitive	0	0
pDU3306	Noncomplementing	Sensitive	0	0
pDU3318	Noncomplementing	Sensitive	0	0

[a] The frequency of recombination is the number of mercuric-resistant colonies formed per diploid cell plated (average of two independent experiments). Stable strains diploid for the two plasmids were obtained by selecting for the transfer of the pDU202*mer*::Tn*801* plasmids from host cell DU1040 into pRR132 or pRR134 host cell KP245 by selecting for Cm^r [Pro^+ Thi^+] recombinants (see text). These plasmid-diploid cells were then plated onto nutrient agar containing 55 μM Hg^{2+} to measure recombination (or complementation) frequency. A value of zero means $<1 \times 10^{-8}$.

[b] The high frequency of mercuric-resistant colonies was probably due to complementation.

[c] NT, Not tested because of high reversion frequencies which would obscure recombination.

in some cases the order of the mutations was not established. Of the three mutants that had insertions located in the *Eco*RI fragment I portion of the *merA* gene, pDU3325 and pDU3328 were distinct since they recombined with each other. The other mutant (pDU3315) was shown by restriction enzyme analysis to have a deletion confined to *Eco*RI fragment I, which probably overlaps the pDU3325 and pDU3328 insertion sites. Three distinct sites were mapped in the *Eco*RI fragment H portion of the *merA* gene (Table 5 and Fig. 3). Mutant pDU3326 was considered to be the most distal of those in *Eco*RI-H because it formed recombinants with the deletions pDU3308 and pDU3327. The order of pDU3316 and the group containing pDU3302, pDU3319, and pDU3320 was not established by these tests. One further mutant (pDU3303) was located in the *Eco*RI-H part of the reductase gene, but further mapping was not performed because pDU3303 reverted at too high a frequency to allow detection of recombination. However, studies with deletion mutations derived from this strain suggest that the pDU3303 insertion site is in the proximal end of the *merA* gene (12).

The naturally occurring plasmid R1 does not confer mercury resistance, but it is known to have extensive nucleotide sequence homology with plasmid R100 (17, 40). R1 contains a Tn*A*-inactivated *mer* operon. pDU3305 and R1 had closely linked *mer* mutations, which were separable by two genetic criteria: (i) they recombined with each other at a frequency of about 5×10^{-7} per donor cell; and (ii) deletions pDU3304, pDU3322, and pDU3323 formed recombinants with R1 but not with pDU3305 (data not shown). Plasmid pDU3317 formed mercuric-resistant recombinants with both plasmid pDU3305 and plasmid R1. There is an apparent contradiction in the map shown in Fig. 3. The deletions of pDU3304, pDU3322, and pDU3323 cannot extend into *merT* or the *op* region because they showed the hypersensitive phenotype and

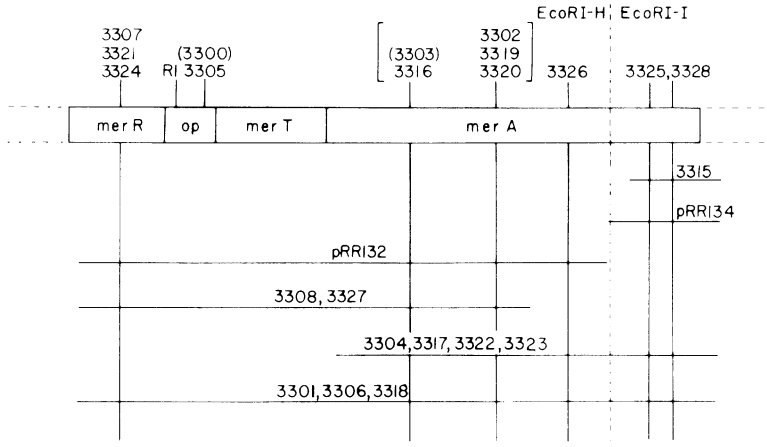

FIG. 3. *Deletion map of the mer region of pDU202. The single horizontal lines represent the deletion mutations. The vertical dashed line is the position of the EcoRI cleavage site separating fragments H and I. The vertical lines are the insertions that behave as point mutations. The relative positions of the mutant numbers of parentheses are not known. The mer genes are merR (the trans-acting regulatory gene), op (the putative operator-promoter site deduced from complementation studies), merT (the gene governing the hyperbinding of Hg^{2+}), and merA (the structural gene for the mercuric reductase enzyme). pRR132 and pRR134 are recombinant plasmids carrying the cloned NR1 EcoRI fragments H and I, respectively (32). They can be considered equivalent to deletions of the R100 DNA that they do not carry.*

should therefore continue to express the *merT* gene. Yet it was not possible to detect recombination between these three mutants and pDU3305. This could be due to the reversion level of pDU3305 (Table 4), which might mask a low frequency of recombination.

Complementation with mutants of plasmid R828. The complementation tests between pDU202 mutants were hampered by the incompatibility of the two IncFII group plasmids. Similar tests were run between the *mer*::Tn*801* mutants of pDU202 and mutants of the IncL plasmid R828 (38). Mutants of strain J53(R828) that had lost mercuric ion resistance were obtained with nitrosoguanidine and with ethyl methane sulfonate. These had simultaneously become sensitive to merbromin and fluoresceinmercuric acetate (as were the pDU202*mer*::Tn*801* mutants) and were also sensitive to phenylmercuric acetate and thimerosal (organomercurials to which plasmid R828, but not R100-1, confers resistance [46]). The additional organomercurial resistances of R828 are ascribed to an additional gene(s) determining the synthesis of organomercurial hydrolase enzyme(s) (39). Mutants of plasmid R828, such as pTK4, retained approximately 1 to 2% of wild-type mercuric reductase activity and complemented the group A (*merR*) mutant plasmids pDU3307, pDU3321, and pDU3324 to full resistance to Hg^{2+}, merbromin, and fluoresceinmercuric acetate. The pTK4/pDU202*merR*::Tn*801* plasmid diploids remained sensitive to thimerosal, however, and were only partially resistant to phenylmercuric acetate. pTK4 did not complement group B hypersensitive mutants pDU3315 and pDU3302 (plasmid diploids remained hypersensitive to Hg^{2+}) and did not complement the group B sensitive mutants pDU3300 and pDU3305. Volatilization assays of mercury loss from Hg^{2+} and organomercurials were consistent with the interpretation that the mutation in R828 derivative pTK4 (and seven similar mutant plasmids) occurred in a *cis*-acting regulatory region. pTK4 was able to provide the *trans*-acting *merR* function missing in group A pDU202*mer*::Tn*801* mutants. It was surprising that, in the screening for mercuric-sensitive strains among 20,000 survivors of mutagenesis, 8 sensitive class B mutants were isolated from R828, but no hypersensitive class B mutants or microconstitutive class A mutants were found. Further genetic studies using transposon mutagenesis with plasmid R828 are required.

Orientation of Tn*801* in pDU202. One can determine the orientation of a transposon inserted in a conjugative plasmid by measuring chromosome mobilization from a strain in which the same transposon is also carried in the host chromosome (15, 24). Transfer of DNA during conjugation is initiated at the plasmid origin of transfer sequence (*oriT*) and proceeds in one direction only (47). The high-frequency mobilization of chromosomal genes located on one side of the transposon is facilitated by the occasional *recA*-dependent recombination event which oc-

curs within the homologous transposons in the chromosome and in the plasmid. Table 6 shows that some pDU202*mer*::Tn*801* elements preferentially mobilized *trp* and others mobilized *his* from UB1731, a strain with Tn*A* inserted in the chromosome between these loci. We conclude that Tn*801* had inserted into the *mer* region in both orientations in different plasmids as diagrammed in Fig. 4.

Analysis of pDU202*mer*::Tn*801* plasmid DNA with restriction endonuclease *Eco*RI. R100 and R100-1 are cleaved into 13 fragments of molecular weight greater than 0.5×10^6 by restriction endonuclease *Eco*RI (45). These fragments are referred to in order of decreasing size as A through M. pDU202 is a tetracycline-sensitive mutant of R100-1 which has lost about 4.8 megadaltons (Mdal) of DNA in the *tet* region of the plasmid (M. A. Kehoe, personal communication). The *Eco*RI cleavage site in the tetracycline resistance determinant Tn*10* was removed by that deletion. This resulted in the fusion of fragments A and D to form fragment A–D (16 Mdal) in pDU202. The *Eco*RI fragments of R100 have been mapped, and in many cases plasmid markers have been assigned to them (27, 30, 45). The *mer* genes are known to be located on fragments H and I (27, 30, 32). Tn*801* is a 3.2-Mdal transposition element (4) which does not have an *Eco*RI-susceptible site (19). The insertion of Tn*801* into the *mer* genes of pDU202 should lead to the disappearance of either *Eco*RI fragment H (3.2 Mdal) or *Eco*RI fragment I (2.7 Mdal), coupled with the appearance of a single new *Eco*RI fragment which is 3.2 Mdal larger. The loss of both *Eco*RI fragments might indicate that a deletion has also occurred. Similarly, a deletion confined to one *Eco*RI fragment would be indicated if the new fragment were smaller than the size expected from the combined molecular weights of Tn*801* and the fragment into which Tn*801* had been inserted.

(i) Insertions into *Eco*RI fragment I. As expected from genetic experiments (Table 5), plasmids pDU3315, pDU3325, and pDU3328 lost band *Eco*RI-I and gained a new fragment (Table 7). The new fragments in pDU3325 and pDU3328 had the expected molecular weight of about 5.6×10^6. However, the new fragment in pDU3315 was 4.0 Mdal in size, indicating that a deletion had occurred in addition to the insertion of Tn*801*. This deletion did not extend beyond the *Eco*RI-H–*Eco*RI-I junction point (Fig. 4).

(ii) Insertions into *Eco*RI fragment H. Most of the mutants were shown to have Tn*801* insertions in *Eco*RI fragment H (Table 7). For the six hypersensitive mutants listed at the top of Table 7 and all five sensitive microconstitutive mutants, the *Eco*RI-H fragment was missing from the restriction endonuclease patterns and replaced by a new fragment of about 6.1 Mdal, the expected addition fragment of *Eco*RI-H and Tn*801*. These 11 mutants are considered to have arisen from insertion of Tn*801* into the three different genes on the *Eco*RI-H fragment without any detectable deletion of DNA.

(iii) Mutations involving a deletion in addition to Tn*801* insertion. The deletions involving the *Eco*RI-H fragment can be conveniently divided into those confined to *Eco*RI fragment H and those involving more than one *Eco*RI fragment. Where more than one pDU202 *Eco*RI fragment was lost, the new band was assumed to be composed of Tn*801* fused to the residue of the two flanking *Eco*RI fragments. It was also assumed that the Tn*801* insertion was immediately adjacent to the material deleted. Two mutants (pDU3308 and pDU3327) were shown by genetic recombination tests (Table 5) to be limited to the *Eco*RI-H portion of the *mer*

TABLE 6. *Orientation of the Tn801 insertion in pDU202*

Plasmid mutant	Frequency of recombination[a]		Tn*801* orientation
	Trp$^+$	His$^+$	
pDU3305, pDU3307, pDU3308, pDU3315, pDU3316, pDU3317, pDU3318, pDU3319, pDU3321, pDU3322, pDU3324, pDU3325, pDU3326, pDU3327, and pDU3328	1×10^{-4} to 4×10^{-3}	$\leq 1 \times 10^{-8}$ to 5×10^{-6}	I
pDU3300, pDU3301, pDU3302, pDU3303, pDU3304, pDU3306, pDU3320, and pDU3323	$\leq 10^{-8}$	2×10^{-5} to 1×10^{-4}	II

[a] The numbers are the frequencies of recombination per donor cell in mating experiments between UB1731 carrying pDU202*mer*::Tn*801* mutants and the recipient strain JC3272, as described in the text. Values were normalized by subtracting the spontaneous level of mobilization of *his*$^+$ and of *trp*$^+$ from the UB1731 donor by plasmid pDU202 not carrying Tn*801*.

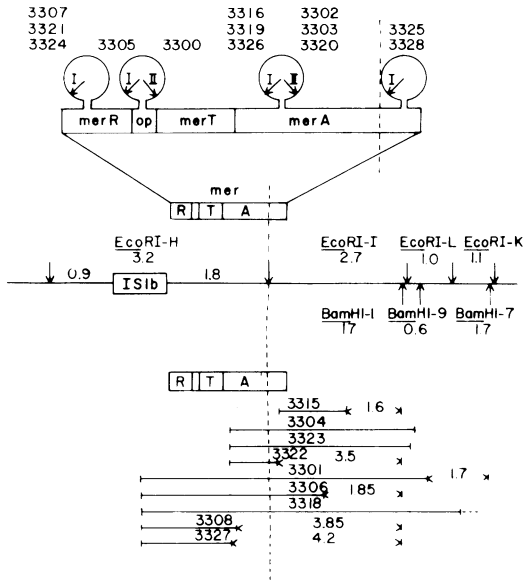

FIG. 4. *Map of the mutational sites in the mer region of pDU202. The top half of the figure depicts the positions of the point insertion mutations used to map the mer genes, as described in the text. The circles represent the Tn801 transposons. The arrows within the circles indicate the position of the BamHI cleavage site carried by Tn801 when the element is inserted in orientation I or II. In the lower half of the map the vertical arrows indicate the positions of the endonuclease cleavage sites: ↓, EcoRI; ↑, BamHI. The figures below the fragment designations are the molecular weights (×10⁶) of the DNA fragments generated by endonuclease cleavage of pDU202 DNA. The position of IS1b was taken from references 5, 29, and 35. The horizontal lines are the estimated positions and extent of the deletions. It should be noted that each mutant probably contains an intact Tn801 element located adjacent to the deleted material. The numbers between the arrows (< >) are the estimated distances from the transposon to the nearest BamHI cleavage site.*

operon. The restriction enzyme analysis indicated that only *Eco*RI fragment H was missing and that a new fragment appeared with a molecular weight of 5.0×10^6 (which comigrated with fragment E).

Mutants pDU3306 and pDU3322 had lost both *Eco*RI fragments H and I. No new *Eco*RI band was visible on the gels of pDU3322 DNA, but we suspect that the fusion fragment comigrated with the *Eco*RI fragment B-C double band. Therefore, the fusion of fragments H and I occurred by a deletion of 1.1 to 1.6 Mdal along with the insertion of Tn*801*. pDU3306 had a larger (3.4 Mdal) deletion covering the entire *mer* region.

Two other mutants (pDU3304 and pDU3323) which retained the ability to complement class A mutants suffered larger deletions involving *Eco*RI fragments H, I, and L (Table 7).

Two mutants lost still larger amounts of DNA after Tn*801* insertion. pDU3318 lost 7.05 Mdal, covering *Eco*RI fragments H, I, L, and K, whereas pDU3301 had a 6.85-Mdal deletion spanning *Eco*RI fragments H, I, and L.

Analysis of pDU202*mer*::Tn*801* plasmid DNA with restriction endonuclease *Bam*HI. Endonuclease *Bam*HI cleaves R100 into nine fragments of molecular weight greater than 0.5×10^6. They are referred to as *Bam*HI-1 through *Bam*HI-9 in order of decreasing size. The fragments have been mapped, and their positions with respect to the R100 *Eco*RI fragments are known (34). pDU202 is a tetracycline-sensitive deletion mutant of R100-1 with loss of pDU202 *Bam*HI fragments 4 and 5 to form fusion fragment *Bam*HI-4-5 (about 6.9 Mdal). The *mer* region of R100-1 is located entirely within the largest *Bam*HI fragment, fragment 1. Tn*801* carries a single *Bam*HI cleavage site. The insertion of *Tn*801 into *Bam*HI-1 followed by cleavage of the recombinant plasmid DNA by nuclease *Bam*HI generated two new fragments, one composed of the larger piece of *Bam*HI fragment 1 plus a section of Tn*801*, and the other consisting of the smaller piece of fragment 1 fused to the other Tn*801* segment. As the *Bam*HI site on Tn*801* is located asymmetrically, cleaving the element into segments of about 1.1 and 2.1 Mdal (36), the sizes of the two new *Bam*HI fragments of the recombinant plasmids depend both on the position and on the orientation of the inserted Tn*801*. In practice, it was only possible to measure accurately the size of the smaller of the new *Bam*HI fragments.

*Bam*HI analysis of pDU202*mer*::Tn*801* mutants with Tn*801* inserted in orientation I (Table 6) provided useful information about the locations of the inactivated genes. The three class A plasmids (pDU3307, pDU3321, and pDU3324) each had Tn*801* in orientation I (Fig. 4). The smaller new *Bam*HI fragments comigrated with the pDU202 *Bam*HI fusion fragment 4-5 and therefore had molecular weights of about 6.7×10^6. With class B plasmid pDU3305, the new *Bam*HI fragment was 6.4 Mdal. The Tn*801* insertion in this case must be located closer by 0.3 Mdal to the *Bam*HI site separating fragments *Bam*HI-1 and *Bam*HI-9 than Tn*801* in pDU3307. Mutant pDU3300 was located in the same region as pDU3305, but had orientation II. It yielded a smaller *Bam*HI fragment of 5.05 Mdal.

The hypersensitive mutant sites were subdivided into proximal and distal sectors by an

TABLE 7. *Restriction endonuclease analysis of pDU202mer::Tn801 plasmid DNA*

Plasmid mutant	*Eco*RI expt[a]		*Bam*HI expt[a]		
	pDU202 fragment(s) missing	Size of new fragment (Mdal)	pDU202 fragment(s) missing	Size of smaller new fragment (Mdal)	Size of deletion (Mdal)[b]
Hypersensitive class					
pDU3302	H	6.1	1	4.6	
pDU3303	H	6.1	1	4.45[c]	
pDU3316	H	6.1	1	6.0	
pDU3319	H	6.2	1	6.0[c]	
pDU3320	H	6.1	1	4.6	
pDU3326	H	6.3	1	5.7	
pDU3315	I	4.0	1	3.6	1.6–1.9
pDU3325	I	5.6	1	5.5[c]	
pDU3328	I	5.45	1	5.3	
Microconstitutive sensitive class					
pDU3307	H	6.1	1	6.7[c]	
pDU3321	H	6.1	1	6.7	
pDU3324	H	6.1	1	6.7	
pDU3300	H	6.0	1	5.05[c]	
pDU3305	H	5.9	1	6.4[c]	
Complementing deletions					
pDU3304	H, I, L	5.4	1, 9	0.91	4.7
pDU3323	H, I, L	5.75	1, 9	0.98	4.35
pDU3322	H, I	7.5–8.0	1	5.5	1.1–1.6
Noncomplementing deletions					
pDU3301	H, I, L	3.35	1, 7, 9	2.7	6.85
pDU3306	H, I	5.7	1	2.85	3.4
pDU3308	H	5.0	1	5.85	1.4
pDU3318	H, I, L, K	4.15	ND	ND	7.05
pDU3327	H	5.0	1	6.2	1.4

[a] The limit of accuracy of the estimates of the sizes of the new fragments whose molecular weights were greater than 4.0×10^6 was about $\pm 0.3 \times 10^6$. ND, Not done. pDU3317 was not analyzed in these experiments.

[b] The size of a deletion was estimated by subtracting the size of the new *Eco*RI fragment from the sum of the molecular weights of the fragments involved in the deletion plus Tn*801*.

[c] These values were obtained from at least four different digestion experiments performed on two separate plasmid DNA preparations.

*Eco*RI cleavage site. Proximal Tn*801* insertions in orientation I generated new *Bam*HI fragments of 5.7 Mdal (pDU3326) to 6.0 Mdal. That pDU3326 had the smallest *Bam*HI fusion fragment of the orientation I insertions in the proximal region of the *merA* gene confirmed the genetic data that this was the closest mutational site to the *Eco*RI-H-I cleavage point (Fig. 3). As expected, the orientation II insertions in this part of the *merA* gene formed smaller *Bam*HI fragments (pDU3302, pDU3303, and pDU3320). Two orientation I insertions occurred in the distal (*Eco*RI-I) part of the *mer* region. pDU3328 had a new *Bam*HI fragment of 5.3 Mdal, whereas the *Bam*HI fragment of pDU3325 was slightly larger at 5.5 Mdal. This suggests that the pDU3325 insertion may be closer to the *Eco*RI site (Fig. 3).

In summary, the sizes of the newly formed *Bam*HI fragments generated by the insertion of Tn*801* in the *mer* region allowed the *mer* genes to be ordered and placed on the pDU202 map with respect to the *Eco*RI site in the mercuric reductase gene and the reference *Bam*HI site separating fragments 1 and 9. Thus, *merR*, which was 6.7 Mdal from the reference *Bam*HI site, was the closest to IS*1*b, whereas *merA* was located closest to the reference point.

The DNA of the pDU202*mer*::Tn*801* deletion mutants was also analyzed with *Bam*HI. Several of the deletions were confined to the large pDU202 *Bam*HI-1 fragment. In each case, the new *Bam*HI fragment that appeared in the gels was assumed to be composed of either 1 or 2 Mdal of Tn*801* DNA (depending on the orientation of the transposon) fused to the remnant of fragment *Bam*HI-1. Thus, the distance from the transposon to the *Bam*HI site separating fragments 1 and 9 could be estimated (Fig. 4). Mutant pDU3315 generated a new *Bam*HI frag-

ment of 3.6 Mdal. Since the transposon was inserted in orientation I (and assuming both that the deletion was contiguous to the transposon and that it did not include deleted transposon sequences [33]), then the right-hand beginning of the deletion in pDU3315 is located 1.6 Mdal (3.6 − 2.0 Mdal) from the *Bam*HI-1-9 reference point (Fig. 4). The left-hand end of the deletion in pDU3315 could be estimated both from the *Eco*RI restriction endonuclease data showing a deletion of approximately 1.9 Mdal entirely within the *Eco*RI-I fragment and from the genetic recombinational analysis (showing that pDU3315 did not give rise to mercuric-resistant recombinants with pDU3328 and pDU3325). pDU3322 formed a new *Bam*HI fragment of 5.5 Mdal, with the Tn*801* insertion being about 3.5 Mdal from the reference *Bam*HI site. This must be an overestimation since there is less than 2.7 Mdal of pDU202 DNA between the *Eco*RI site in the reductase gene and the *Bam*HI-1-9 reference site. Perhaps the Tn*801* element is located further to the left and is not contiguous with the deleted material. Similar analysis led to the mapping of the transposons of mutants pDU3306, pDU3308, and pDU3327. The 3.4-Mdal length of the deletion in pDU3306 (from the *Eco*RI enzyme analysis) would place the left-hand end of this deletion 2.5 Mdal into the *Eco*RI-H fragment and (within the errors of these measurements) on the resistance transfer factor side of the IS*1*b sequence. This is consistent with the failure of pDU3306 to recombine with any of the other mercuric-sensitive mutants. The deletion in pDU3308 started slightly further to the right (Fig. 4) than the deletion in pDU3327. Both pDU3308 and pDU3327 probably have left-hand deletion endpoints in the IS*1*b sequence. The right-hand deletion endpoints of plasmids pDU3308 and pDU3327 extended into the reductase gene, since these mutant plasmids failed to give rise to recombinants with the leftmost of the hypersensitive insertion mutations (Fig. 3).

Other strains had deletions that removed the right end of the *Bam*HI-1 fragment, as well as fragment *Bam*HI-9 or fragments *Bam*HI-9 and *Bam*HI-7 (Fig. 4 and Table 7). Analysis of pDU3304 did not reveal a new *Bam*HI fragment, and it seemed likely that a new band comigrated with pDU202 fragment *Bam*HI-8. The deletion endpoint would then be located very close to the *Bam*HI site which separates fragments *Bam*HI-9 and *Bam*HI-7. pDU3323 had a similar deletion. The pDU3301 mutation removed pDU202 *Bam*HI fragments 1, 7, and 9 (Table 7). The site of Tn*801* was estimated to be 1.7 Mdal from the reference *Bam*HI site between *Bam*HI fragments 6 and 7 (Fig. 4).

DISCUSSION

Tn*801*-generated insertion and deletion mutations in the *mer* genes of plasmid pDU202 were analyzed phenotypically, genetically, and with restriction endonucleases. Tn*801* insertions located in *merA* (Fig. 3 and 4), the gene for the reductase enzyme, rendered the host cells hypersensitive to mercurials. The strains rapidly bound Hg^{2+} added to the culture medium. We postulated from these results that these mutants continue to express a high-affinity mercury uptake function (governed by a gene *merT*), which normally brings Hg^{2+} through the outer layers of the *E. coli* cell to a site in the cytoplasm or on the inner cytoplasmic membrane surface where it is accessible to the mercuric reductase enzyme. Recent experiments with λ *mer* phages have identified three major Hg^{2+}-inducible proteins (8). One corresponds to the 68,000-dalton subunit of the enzyme mercuric reductase. Either of the other two proteins (11,500 and 8,500 daltons) would be a possible candidate for the *merT* product.

The expression of transport in the absence of reductase results in the accumulation of toxic Hg^{2+} within the cells. The cloned R100 *Eco*RI-H fragment functioned in a similar fashion, conferring hypersensitivity to mercurials and rapid Hg^{2+} binding by the host cell (32). Cloned fragment *Eco*RI-H also complemented class A pDU202*mer*::Tn*801* mutants. These results suggest the existence of a *trans*-acting regulatory gene (*merR*), which is nonfunctional in class A mutants, and a *cis*-acting regulatory site, which is defective in the two class B microconstitutive mutants pDU3300 and pDU3305, as well as in wild-type plasmid R1. These three plasmids have properties consistent with operator-promoter mutants, although more work will be required to establish this hypothesis. The *merR* gene product is a positive *trans*-acting regulatory element. In its absence, the *mer* operon was only expressed at a low level, which was sufficient to give about 2% of the normal level of reductase, but not high enough to confer measurable resistance. The *merR* regulatory protein may also function to repress *mer* operon function down from the nonregulated 2% level to less than 0.1% (Table 3). Activation by Hg^{2+} (directly or indirectly) would convert the regulatory protein into a positively acting form that allows maximum (100%) synthesis from the operon. This might be analogous to the functioning of the *araC* protein and the arabinose system promoter on the chromosome of *E. coli*. Regulation of the *ara* system functions both positively and negatively, and understanding of the *ara* system is well advanced at the in vitro and sequence

levels (41). Savageau (37) has argued that a positively functioning regulatory system (like *ara*) would be selected for substrates frequently encountered in nature (like Hg^{2+}), whereas negatively regulated systems would be preferred for rarer substances, such as lactose and perhaps those antibiotics to which resistances are governed by inducible plasmid systems.

Tn*801* inserted preferentially into the *mer* region of the plasmid. Thus, 2.5% of pDU202::Tn*801* insertions were Mer^s, whereas less than 0.1% were defective in *spc*, *sul*, *chl*, or *tra*. Tn*3*, which is closely related to Tn*801*, was previously shown to transpose preferentially into *Eco*RI fragments 3 and 9 of R6-5 (26), a plasmid which shares extensive sequence homology with R100-1 (40). In the R6-5 study, 7 of 23 Tn*3* insertions occurred in *Eco*RI fragment 9 (which corresponds to fragment *Eco*RI-H of R100-1). The mercuric resistance phenotype of these mutants was not tested. Although Tn*A* inserts nonrandomly, the fine structure analysis of the Tn*801* insertion sites within the *mer* region revealed that the transposon inserted at more than one position.

Nearly one-half of the *mer* mutations that were caused by the insertion of Tn*801* involved a further deletion of part or all of the *mer* region. Tn*A*-promoted deletions have been reported previously, both during the initial insertion event (42) and after the Tn*A* was resident in the DNA (20). This highlights a potential danger of using Tn*A* to isolate insertion mutations without subsequent fine structure analysis. It often has been assumed that transposon-promoted insertion mutations are precise events which behave as point mutations (24). However, only 5 of the 23 pDU202*mer*::Tn*801* mutants reverted to mercury resistance at measurable frequencies.

R1, an R plasmid discovered in England in 1965 (28), differs in the r-determinant region from R100 (which originated in Japan) by two insertions, one conferring ampicillin resistance (Tn*3*) and the other causing kanamycin resistance. Otherwise the r-determinant sequences of R1 and R100 are homologous (25, 40). However, R1 does not confer resistance to Hg^{2+}. It was shown here that R1 possesses a *merA* gene that is expressed microconstitutively. The Tn*3* element on R1 appears to be located in the same gene as, and very close to, the pDU3305 insertion site. Thus, the Tn*A* element was acquired by a transposition event in nature similar to that of the Tn*801* insertions in the laboratory. The region of R1 between Tn*A* and IS*1* (corresponding to the area where the *merR* gene is located) codes for a 13,500-dalton protein (17) which may correspond to a minor polypeptide of this size identified by Dempsey et al. (8).

We attempted to place limits on the length of the *mer* region and on its two endpoints by using the *Bam*HI restriction endonuclease data plus other available information. Since an isolated RTF-Tc element (*R*esistance *T*ransfer *F*actor-*T*etracycline resistance [5]) did not give mercuric-resistant recombinants with any of the *mer*::Tn*801* mutants (Foster, unpublished data), the *mer* region must begin on the *r*-determinant side of IS*1*b, as shown in Fig. 4. However, the *Bam*HI fragment sizes from the three *merR* mutants were all 6.7 Mdal (Table 7), which (after subtraction of the 2.1-Mdal portion of Tn*801* and the 2.7 Mdal from the *Bam*HI-1-9 cleavage site to the *Eco*RI-H-I cleavage site) would place the Tn*801* insertion site in *merR* at a position 1.9 Mdal to the left of the *Eco*RI site. The best available data (30, 34, 35) allows 1.8 Mdal of DNA between IS*1*b and the *Eco*RI site (Fig. 4). Thus, within the current limits of resolution, the *merR* gene closely abuts the IS*1*b insertion element. There is a problem with the right-hand terminus of *mer* from the *Eco*RI and *Bam*HI analysis. The *Eco*RI endonuclease analysis (Table 7) showed that the Tn*801* insertions in the three right-most mutants occurred in *Eco*RI fragment I, demonstrating that the *merA* gene straddled the *Eco*RI-H-I cleavage site. Yet, the *Bam*HI fragment from plasmid pDU3328 (which appeared to have its Tn*801* insertion site furthest to the right) was 5.3 Mdal in size. This would place the insertion site 3.2 Mdal from the *Bam*HI-1-9 cleavage site (Fig. 4), whereas there is only 2.7 Mdal of DNA between that site and the *Eco*RI site in the *merA* gene. The *Bam*HI fragment appeared too large for the concluded position of the transposon, probably due to the inherent inaccuracy in measuring DNA fragments in the gel system used. The positions of Tn*801* insertion in the outermost mutants provided a minimal estimate for the size of the *mer* region of about 2 Mdal. This compares well with the expected molecular weight of DNA needed to code for the known Hg^{2+} inducible polypeptides (8). Polypeptides that are 68,000, 13,500, and 8,500 daltons would be coded by 1.4 Mdal of DNA, with the *merA* reductase gene itself requiring 1.2 Mdal of DNA. Closer placing of the boundaries of the *mer* region might come from heteroduplex DNA analysis of the present plasmid mutants. However it seems more likely that studies of progressively smaller cloned fragments of the *mer* region will be informative. A start in that direction is reported in an accompanying paper (32).

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