Deletions in the r-Determinant *mer* Region of Plasmid R100-1 Selected for Loss of Mercury Hypersensitivity

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A mutant of plasmid R100-1, which conferred cellular hypersensitivity to Hg^{2+} because of the insertion of Tn*801* (Tn*A*) into the gene determining synthesis of mercuric reductase enzyme, allowed further mutational events to be selected which resulted in either reversion to Hg^{2+} resistance (characteristic of plasmid R100-1) or sensitivity at a level characteristic of plasmidless strains. Restriction endonuclease *Eco*RI and *Bam*HI analysis showed that reversion to resistance resulted from loss of Tn*A* from the R100-1 *mer*::Tn*801* plasmid, whereas the change from hypersensitivity to sensitivity to Hg^{2+} usually resulted from deletion of part or all of Tn*801* plus plasmid deoxyribonucleic acid sequences corresponding to the operator-proximal end of the *mer* operon.

The drug resistance-determinant (r-determinant) region of plasmid R100 (also known as NR1 or R222) carries genes which code for resistance to mercuric ions, streptomycin-spectinomycin, sulfonamides, fusidic acid, and chloramphenicol. Each of these loci has been mapped by cloning of restriction endonuclease *Eco*RI fragments and by analysis of deletion mutants (2, 5, 7, 13). The *mer* genes are located close to one insertion sequence IS*1* boundary of the r-determinant and span fragments *Eco*RI-H and *Eco*RI-I (5, 7). Analysis of Tn*801* insertion mutants in the *mer* region of a derivative of R100-1 led to the following hypothesis for the structure and control of the *mer* genes (3). At least two structural genes are required for the expression of mercuric salts resistance; *merT* determines a transport function, which directs extracellular Hg^{2+} into the cell, where it can be reduced by the enzyme determined by the *merA* gene, the NADPH-dependent mercuric reductase. Both genes are expressed in an inducible operon with the direction of transcription being *merT-merA*. The presence of a *trans*-acting regulatory element determined by the *merR* gene is required for the expression of *merT* and *merA*.

The Tn*801* mutations located in *merA* confer hypersensitivity to mercurials upon their host cells (3). Cells carrying these mutants are four to seven times more sensitive to Hg^{2+} than the R^- strain and bind three to five times as much Hg^{2+} as do sensitive cells (in a process that requires induction by subtoxic levels of Hg^{2+} [3]). In this paper, the properties of seven different mutants derived from the hypersensitive plasmid pDU3303 (R100-1 *merA*::Tn*801*) are re-

ported. Derivatives of pDU3303 which are capable of growing on slightly higher concentrations of Hg^{2+} were selected. Either the entire plasmid was lost or a secondary mutation which abolished the transport function (*merT*) had occurred. Some of these derivatives have extensive deletions spanning most of the r-determinant, whereas others have shorter deletions between Tn*801* and IS*1b*.

The derivation and properties of the *Escherichia coli* bacterial strains JC3272, UB1731, and UB258 are given in an accompanying paper (3). Strain JSR0 *pro met* was sent by J. A. Shapiro. The pDU202*mer*::Tn*801* plasmids pDU3303 and pDU3304, as well as plasmids used in the mapping experiments, are also described in reference 3. The derivatives of pDU3303 (*merA*::Tn*801*) which had lost hypersensitivity to Hg^{2+} are listed in Table 1.

Culture media, resistance level determinations, complementation tests, recombination analysis, chromosome mobilization tests, isolation of plasmid DNA, and analysis with restriction endonucleases are all described in an accompanying paper (3).

The *merA*::Tn*801* plasmids pDU3303 and pDU3304 conferred hypersensitivity to $HgCl_2$ upon host *E. coli* cells (3). During experiments with pDU202*merA*::Tn*801* mutants, clones arose which no longer conferred the hypersensitive phenotype. The frequency at which these more resistant derivatives occurred in cultures carrying pDU3303 and pDU3304 was about 4.5 × 10^{-4}. Cultures of strain JC3272 carrying plasmid pDU3303 or pDU3304 were grown overnight with shaking in broth before being diluted and

TABLE 1. *Characterization of derivatives of pDU3303 which no longer confer hypersensitivity to mercuric chloride[a]*

Phenotype	No. isolated	No. of independent mutants	Representative plasmid(s)
Mer^s Amp^s Sul^s Spc^c Chl^r	4	4	pDU3349
Mer^s Amp^s Sul^s Spc^c Chl^r	6	4	pDU3353
Mer^s Amp^r Sul^r Spc^c Chl^r	51	10[b]	pDU3360, pDU3361, pDU3367
Mer^s Amp^s Sul^r Spc^c Chl^r	28	10	pDU3356
Mer^r Amp^s Sul^r Spc^c Chl^r	1	1	pDU3330

[a] Nine colonies each from 10 independent cultures of strain JC3272(pDU3303) were obtained by plating onto nutrient agar containing 20 μg of chloramphenicol per ml and 4 μM HgCl$_2$.

[b] Of the 10 independent derivatives in this class, 2 lost the Amp^r marker upon transfer to host cell JSR0 with selection for Chl^r [His$^+$ Trp$^+$ Lys$^+$].

plated onto nutrient agar containing either chloramphenicol plus 4 μM HgCl$_2$ or HgCl$_2$ alone (4 μM Hg^{2+} permits growth of mercury-sensitive but not mercury-hypersensitive strains). The colonies which grew on Hg^{2+} alone were replica plated directly onto chloramphenicol-containing agar. About 85% of the colonies had lost all of the pDU202 markers. These were presumed either to have been cured of the entire plasmid or to have segregated the entire r-determinant region.

Colonies that grew on Hg^{2+} plus chloramphenicol agar appeared at a frequency of 6 × 10^{-5}. These were picked and stabbed onto the same medium, grown overnight, and replica plated to score ampicillin, sulfonamide, and spectinomycin sensitivities (Table 1). Many of these clones had lost Amp^r from Tn801, and some had lost Sul^r or Sul^r and Spc^c from plasmid R100-1. The most frequent class of variants retained the Amp^r determinant, although in 2 of 10 cases tested, the Amp^r (i.e., Tn801) marker was not transferred during conjugation with the other plasmid determinants. Possibly the Amp^r determinant in these donor strains was rescued after excision from the plasmid by integration into the host cell chromosome. This point was not examined further.

Amp^r Mer^s derivatives might have arisen by deletions which removed the *merT* gene located proximal to Tn801 in *merA*. Plasmid pDU3303 also reverts to mercuric resistance at a frequency of 4 × 10^{-7} (3), with the consequent loss of Tn801. One such derivative (pDU3330) was also isolated during these experiments.

Seven different mutants representing the various phenotypic classes were chosen for further

analysis. Each mutant was tested for Hg^{2+} resistance level, for the synthesis of the mercury-volatilizing and mercury-binding activities, and for chromosome mobilization from strain UB1731 (Table 2). The hypersensitive parental plasmid pDU3303 and the six sensitive derivative plasmids did not confer measurable mercuric reductase activity on the host cells (Table 2). Whereas hypersensitive cells with plasmid pDU3303 bound appreciably more ^{203}Hg^{2+} than did plasmidless sensitive cells (3), the plasmid-harboring sensitive derivatives (Table 2) showed Hg^{2+} binding comparable to that of plasmidless strains (data not shown). The mercuric salts-resistant revertant pDU3330 conferred a normal enzyme level upon the host cells (Table 2), and the synthesis of this activity was inducible (data not shown), as was the case for the parental plasmid (3).

The Amp^s mutants pDU3349, pDU3353, and pDU3356 failed to mobilize the chromosomal *his* genes from TnA-carrying strain UB1731. However, a difference was noted in the frequency of chromosome mobilization by pDU3360 and pDU3367 (both Mer^s Amp^r). pDU3367 transferred *his* at a very low frequency compared with pDU3360 and the parental plasmid pDU3303. This suggested that a deletion of part of the transposon had occurred, which left the β-lactamase gene intact. pDU3303 carries Tn801 with the β-lactamase gene in the sector of the transposon distal with respect to *merT* (3) (Fig. 1). Thus, up to 2 megadaltons (Mdal) of DNA could be removed from the *merT* end of Tn801 without affecting Amp^r.

TABLE 2. *Properties of derivatives of pDU3303 with altered mercuric resistance*

Plasmid	Mercuric resistance (μM)[a]	Volatilization (nmol/min per mg)[a]	Mobilization of chromosomal *his* genes[b]
pDU3303	3	<0.01	4 × 10^{-5}
pDU3349	10	<0.01	<1 × 10^{-8}
pDU3353	10	<0.01	<1 × 10^{-8}
pDU3356	10	<0.04	<1 × 10^{-8}
pDU3361	10	<0.04	NT
pDU3360	10	<0.04	1 × 10^{-5}
pDU3367	10	<0.04	8 × 10^{-7}
pDU3330	75	7.1	<1 × 10^{-8}
pDU202	75	7.1	<1 × 10^{-8}

[a] Resistance level to HgCl$_2$ and whole cell volatilization rates for ^{203}Hg^{2+} were determined as described in reference 3.

[b] Frequencies of recombination per donor cell in mating experiments between strain UB1731 (::TnA) carrying the pDU3303 derivatives and recipient strain JC3272, normalized to account for the very low levels of transfer of *his* from UB1731 by pDU202 as described in detail in reference 3. NT, Not tested.
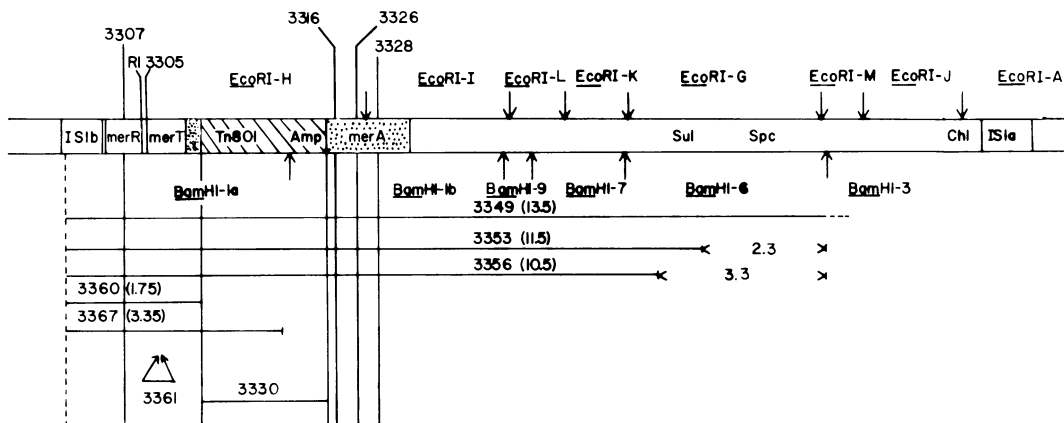
FIG. 1. *Deletion map of the r-determinant region of recombinant plasmid pDU3303. This deletion map incorporates the genetic analysis deduced from recombination and complementation tests with point insertion mutants in merR and merA, as well as analysis of the plasmid DNA with EcoRI and BamHI. ↓, EcoRI cleavage sites from reference 13; ↑, BamHI cleavage sites from reference 10. The locations of the Sul^r, Spc^r, and Chl^r genes were deduced from references 5, 7, and 13. The mapping of the merA, merR, and merT genes is reported in an accompanying paper (3). The vertical lines represent the point Tn801 insertion mutants in mer genes. The cross-hatched area is the position of the Tn801 element in the (stippled) merA gene of pDU3303. Note that the Tn801 element (which is not drawn to scale) introduced an extra BamHI site into the pDU202 r-determinant region. This generated two new bands in plasmid pDU3303, which are called 1a and 1b. The horizontal lines represent the extent of the deletions in different plasmids. The numbers in parenthesis are the sizes of the deletions in megadaltons (Mdal). It was assumed that each deletion started at the left-hand terminus of IS1b.*

Each pDU3303 variant was tested for its ability to complement the *merR* mutant plasmid pDU3307 and for recombination with a series of point Tn*801* insertion mutants which have been mapped (3). The nature of the lesions was confirmed by restriction endonuclease enzyme analysis of the DNA of the mutant plasmids. The results of agarose gel electrophoresis of enzyme-digested DNA are summarized in Table 3.

Only one mutant (pDU3361) retained the ability to complement the *merR* mutant pDU3307, indicating that it continued to express the regulatory *merR* gene. Each of the other mutations were shown to be deletions by restriction endonuclease analysis. Plasmid pDU3361 had acquired an insertion of 0.4 Mdal; and the new *Eco*RI fragment replacing *Eco*RI-H::Tn*801* of plasmid pDU3303 had a molecular weight of 6.8 × 10⁶ (Table 3). This mutant had a *Bam*HI cleavage pattern identical to that of the parental plasmid, pDU3303. Since an insertion of 0.4 Mdal would not noticeably affect the mobility of the large *Bam*HI fragment 1a, but would have been seen in the smaller 1b fragment (4.45 Mdal), this places the point of insertion to the left of the Tn*801* BamHI site (Fig. 1). Since the insertion inactivated the *merT* function, it must have occurred in the *merT* gene or in the adjacent operator-promoter region. However, if the *merT* sequence of mutant pDU3361 remained

TABLE 3. *Endonuclease cleavage patterns with the pDU3303 variants*

Mutant plasmid	Analysis with EcoRI[a]		Size of deletion[b] (Mdal)	Band(s) missing in BamHI analysis[a, c]
	Band(s) missing	Size of new band (Mdal)		
pDU3330	H*	3.2	3.2	1b
pDU3361	H*	6.8	Insertion of 0.4	None detectable
pDU3367	H*	3.05	3.35	None detectable
pDU3360	H*	4.65	1.75	None detectable
pDU3349	H*,I,L,K,G,M	1.8	13.5	1b,9,7,6
pDU3353	H*,I,L,K,G	3.2	11.5	1b,9,7,6
pDU3356	H*,I,L,K,G	4.2	10.5	1b,9,7,6

[a] Restriction endonuclease *Eco*RI and *Bam*HI digestions and agarose gel electrophoresis were carried out as described in reference 3. The pDU3303 *Eco*RI fragment H*(6.4 Mdal) is equivalent to the pDU202 *Eco*RI-H fragment (3.2 Mdal) carrying inserted Tn*801* (3.2 Mdal). The *Bam*HI fragment 1b of pDU3303 had a molecular weight of 4.45 × 10⁶ (3).

[b] The size of the deletion was estimated by subtracting the size of the new *Eco*RI fragment from the molecular weights of the *Eco*RI bands that were missing.

[c] In no case was a new band detected. However, large new bands were not discernible under the conditions of electrophoresis employed because the area at the top of the gels was crowded with several high-molecular-weight *Bam*HI fragments.

intact apart from the insertion, it might have recombined with plasmids R1 and pDU3305. The failure to obtain recombinants in crosses of pDU3361 with R1 and pDU3305 with a detection limit of 5×10^{-7} might be explained by the two closely linked insertions in pDU3361 which prevented the interaction of the homologous sequences. Very low frequencies of recombination could not be measured because of reversion of pDU3305 and the background growth that occurred when large inocula were plated onto Hg^{2+} agar (3).

Mutants pDU3349, pDU3353, and pDU3356 had lost a substantial portion of the r-determinant region spanning the entire *mer* operon, the Tn*801* transposon, and in two cases the Sul^r or the Sul^r and Spc^r genes (Table 1 and Fig. 1). In pDU3349 and pDU3353 a deletion extending as far as fragment *Eco*RI-G was expected since previous studies had mapped the Sul^r and Spc^r genes in this region (5, 7, 13). The Sul^s Spc^s mutant pDU3349 had lost *Eco*RI fragment M as well, with the formation of a new band of 1.8 Mdal which comigrated with *Eco*RI-J. This new band is presumed to be a fusion of the residues of fragments H and M, which would result from a deletion of 13.5 Mdal (Fig. 1). The *Bam*HI analysis confirmed that a large deletion was involved. However, it was not possible to determine whether *Bam*HI fragment 3 was lost.

The Sul^s Spc^r mutant pDU3353 had a shorter deletion of 11.5 Mdal, which removed five *Eco*RI and four *Bam*HI fragments. Mutant pDU3356, which retained Spc^r and Sul^r, had lost the same *Eco*RI and *Bam*HI fragments (Table 3). In this case, 10.5 Mdal of DNA was deleted. If each of the deletions starts on the left at the same position (postulated to be the left-hand end of IS*1*b), then the other endpoints in fragment *Eco*RI-G can be estimated. IS*1*b starts 0.9 Mdal into *Eco*RI fragment H (1, 10). Therefore, the pDU3353 deletion would extend 1.2 Mdal into fragment G, and the pDU3356 deletion would extend 0.2 Mdal (Fig. 1). These estimates would be increased by 0.5 Mdal if the other terminus of IS*1*b was involved.

Two mutants (pDU3360 and pDU3367) representing the common class of sensitive variants of pDU3303 were chosen for study. Both had lost the pDU3303 band H* and gained smaller bands (Table 3). If it is assumed again that the left-hand terminus of IS*1*b is the starting point of the deletions, then it is possible that the 1.75-Mdal pDU3360 deletion extends as far as the beginning of the Tn*801* transposon, whereas the larger pDU3367 deletion of 4.65 Mdal could extend 1.6 Mdal into Tn*801*. Up to 2.0 Mdal could have been lost from Tn*801* inserted in this

orientation without affecting the *Bam*HI cleavage site or Amp^r. Both mutants retained the pDU3303 *Bam*HI fragment 1b (Table 3), indicating that both deletions were confined to fragment 1a.

One mercuric-resistant revertant (pDU3330) was included in the study. As expected, the 6.4-Mdal recombinant fragment H* had reverted to the wild-type *Eco*RI-H fragment of 3.2 Mdal, and the pDU3303 fragment *Bam*HI-1b was lost, indicating that the Tn*801* *Bam*HI cleavage site was removed.

This paper reports a useful method for obtaining deletions in the r-determinant region of plasmids with mercuric resistance determinants. Plasmids with a mutation in the mercuric reductase gene confer the hypersensitivity phenotype upon host cells because these strains continue to express the Hg^{2+} uptake function in the absence of volatilization activity. By selecting for the growth of colonies with greater mercury resistance, additional deletion mutations affecting the uptake function were readily obtained. A procedure recently described by Rownd et al. (12) enabled similar deletion mutations starting in fragment *Eco*RI-G of plasmid R100 to be selected after the transfer of the streptomycin-inactivating *spc-str* gene into a streptomycin-dependent host.

The frequency at which the deletions occurred was 5×10^{-5}. This high frequency is possibly attributable to the closely linked IS*1*b sequence. IS*1* has been implicated in deletion events both in the host chromosome (11) and in R plasmids such as R1 (9) and R100 (6). In addition, Tn*801* might contribute to the instability. The termini of Tn*A* have been implicated in deletion events (3, 4, 8).

The parental plasmid pDU3303 contains Tn*801* in the proximal part of the *merA* gene. The deletion mutants pDU3360 and pDU3367, which retained Amp^r, also retained *mer* DNA distal to the insertion site. From recombination analysis of crosses with point insertion mutants in *merA*, the pDU3303 lesion was found to be the most proximal of the *merA* insertion sites (Fig. 1).

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