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Translation of *merD* in Tn*21*

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All four sequenced examples of the mercury resistance (*mer*) operon of gram-negative bacteria have a promoter-distal reading frame, *merD*, whose removal has little effect on the resistance phenotype and whose translation has not previously been observed. Using *merD-lacZ* protein fusions, we show that *merD* is translated. However, Hg(II)-induced *merD* expression, as measured by β-galactosidase activity and immunoblotting, is 10- to 15-fold lower than that of fusions to the gene immediately preceding it, *merA*.

In Tn*21* and Tn*501*, the mercury resistance locus (*mer*) consists of a regulatory gene (*merR*), mercuric ion [Hg(II)] transport genes (*merT*, *merP*, and *merC* in Tn*21*), and the mercuric reductase gene (*merA*) (for reviews, see references 6 and 25). An additional open reading frame (*merD*) occurs in both Tn*21* and Tn*501* closely juxtaposed to the carboxy terminus of *merA*; however, it was uncertain whether *merD* was expressed and, if so, what its function might be (4, 12, 23). End mapping of *mer* mRNA demonstrated that the *merD* region is transcribed (8). However, there are several factors which could compromise the translation of this open reading frame (10, 11, 19). In Tn*21*, these factors are (i) two tandem Shine-Dalgarno sequences; (ii) a G immediately 5′ to the initiation codon, which is the least common base at that position; and (iii) an unusually GC-rich sequence immediately 3′ to the initiation codon (AGCGCC). Tn*501* *merD* is preceded by only one consensus Shine-Dalgarno sequence; it has an A immediately 5′ to the initiation codon, and the second *merD* codon is AAC. Nevertheless, in neither Tn*21* nor Tn*501* has any protein corresponding to *merD* been observed. The predicted *merD* protein bears considerable resemblance to the regulatory protein *merR*, especially in its amino-terminal helix-turn-helix region (4). In this article we report the first evidence for translation of *merD* obtained by using gene fusion and immunoblotting. We also confirm a phenotype of slightly lower Hg resistance for deletion mutations in *merD*.

Deletion mutations in *merD* and *merD-lacZ* protein fusions. To detect the expression of *merD*, we constructed deletion mutations and *lacZ* protein fusions in this gene. As controls, similar constructs were made in *merA* and immediately downstream of the *mer* operon. We made deletions in *mer* genes by digesting pDB7 (1) with *Bal* 31 for various times and inserting a *Bam*HI linker at each deletion endpoint (18). The resulting *Eco*RI-*Bam*HI fragment of each deletant was used to replace the corresponding fragment of pDG106 (9) (Fig. 1 and 2). We confirmed the endpoint of each deletion by Maxam and Gilbert DNA sequencing (20).

We then fused the *lacZ* gene to each deletion endpoint, either directly or by using *Bgl*II linker to obtain both in-frame and out-of-frame fusions in *merD* (Fig. 2). We made similar fusions in *merA* and downstream of the *mer* operon to be used as positive and negative controls. Double-stranded-DNA sequencing (13) with a 24-mer M13 primer confirmed the *merD-lacZ* and *merA-lacZ* fusion junctions.

Mercury resistance phenotypes of deletion and fusion mutants. Mercury resistance intermediate between that of a wild-type *mer* strain and that of a plasmid-free host strain has been attributed to multicopy *merD*::Tn*5* insertions (23). However, no gene product had previously been detected from the *merD* reading frame (15, 16, 22). The efficiency of plating of strains carrying two of our *merD* deletions and their respective *merD-lacZ* fusions was also intermediate between that of the fully-resistant wild-type plasmid (pDG106) and that of the sensitive host strain (BW11331), although their inhibition zone sizes (2) showed no difference (Table 1). Relevant control deletions and fusions either in *merA* (pIL12 and pIL32) or completely beyond the *mer* operon (pIL8 and pIL58) caused the expected hypersensitivity or resistance to Hg(II) (Table 1). These observations are consistent with the relatively minor role of *merD* in expression of Hg(II) resistance.

Detection of Mer-Lac fusion proteins. We detected the translational product of *merD* indirectly by determining the expression of β-galactosidase from *merD-lacZ* protein fusions (Fig. 3). Exponentially growing cells in fructose minimal medium (21) at 37°C were induced for 20 min with final concentrations of 0.1 μM $HgCl_2$ for sensitive and hypersensitive strains and 3.0 μM $HgCl_2$ for resistant strains. These cultures were used immediately for β-galactosidase assay as described by Miller (21) with a modification of sodium dodecyl sulfate-chloroform lysis. The host strain, out-of-frame *lacZ* fusion strains, and pIL58 showed no increase in *lacZ* activity after $HgCl_2$ induction, whereas the *merD-lacZ* in-frame fusion strains exhibited significant increases in β-galactosidase activity when $HgCl_2$ induced (Table 1). The maximum activity of *merD-lacZ* fusion strains was 10-fold lower than that of the *merA-lacZ* fusion strain. In the uninduced state, only pIL32 (*merA-lacZ*) showed any detectable β-galactosidase activity.

We also observed the fused gene products directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Whole cells (1 ml) from the $HgCl_2$-induced or the uninduced cultures were harvested, suspended with the buffer system of Tabor and Richardson (26), and loaded onto a 7.5% discontinuous sodium dodecyl sulfate-polyacrylamide gel (17). After electrophoresis, proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Inc.), probed with monoclonal anti-β-galactosidase antibody (Promega Biotec), and detected by

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TABLE 1. Phenotypes of strains carrying *mer* deletions and *lacZ* fusions

Plasmid	Deletion and Fusion Map[a]	Genotype[b]	Zone of Inhibition (mm)	Phenotype E.O.P.[c]	Phenotype ß-galactosidase[d] +Hg	Phenotype ß-galactosidase[d] -Hg
pIL8	A 3447 D 3827 4297	*mer*+	13	0.6	nd[e]	nd
pIL58	A D *lacZ*	*mer*+ *'lacZ*	13	0.7	0	0
pIL10	A (D) 3757	*Δ merD'*	13	0.3	nd	nd
pIL30	A (D) *lacZ*	*ΔmerD'-'lacZ* protein fusion	13	0.2	457	0
pIL11	A (D) 3612	*ΔmerD'*	13	0.1	nd	nd
pIL31	A (D) *lacZ*	*ΔmerD'-'lacZ* protein fusion	13	0.3	332	0
pIL12	(A) 3165	*ΔmerA', ΔmerD*	28	0	nd	nd
pIL32	(A) *lacZ*	*ΔmerA'-'lacZ* protein fusion	28	0	4,204	20
Controls:						
pDG106		*mer*+	13	0.6	nd	nd
pDG113		*ΔmerA*	30	0	nd	nd
pDG121		*ΔmerR*	17	0.5	nd	nd
pDU202		*mer*+	12	0.8	nd	nd
Host Strain BW11331[f]		*Δlac*	18	0	0	0

[a] Numbers under each map represent deletion endpoints as in Fig. 1. Numbers above the map are the positions of the 3' end of each gene.
[b] +, Wild-type gene; *'lacZ*, *lacZ* gene missing first 7 codons; Δ, deletion of gene; *ΔmerD'*, deletion of 3' end of *merD*.
[c] The efficiency of plating (E.O.P.) is the ratio of CFU on 50 μM $HgCl_2$-containing L agar divided by the CFU on L agar (for BW11331) or on L agar containing 25 μg of chloramphenicol per ml (for pDU202)(7) or 50 μg of kanamycin per ml (for all other strains).
[d] In Miller units; determinations done four times, each in duplicate. Standard deviation, ≤38%.
[e] nd, Not detected.
[f] Source, Barry Wanner.

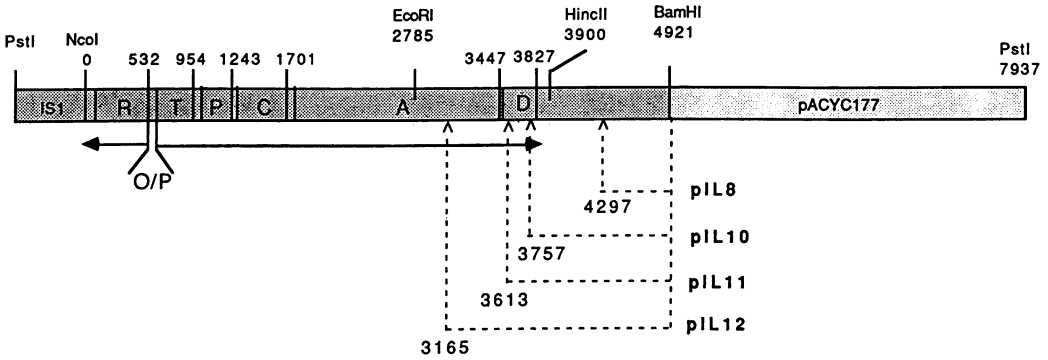

FIG. 1. *mer* operon in pDG106. Divergent transcripts are indicated by solid arrows from the operator-promoter region (O/P) (14). Arrows with broken lines represent deletion endpoints from the *Bam*HI site. Base pair numbering is as in reference 1.

using alkaline phosphatase conjugated antibody (Sigma Chemical Co.) and consequent color development (3). The apparent molecular weight of each fused protein (pIL30, 125,000; pIL31, 120,000; and pIL32, 167,000) was close to that predicted by DNA sequence analysis (pIL30, 125,838; pIL31, 120,673; and pIL32, 167,328). No translational readthrough product from *merA* to *merD* was detected. On the basis of densitometry, the difference in the amounts of fusion proteins MerA-LacZ and MerD-LacZ seen by immunoblotting was approximately 10- to 15-fold (data not shown).

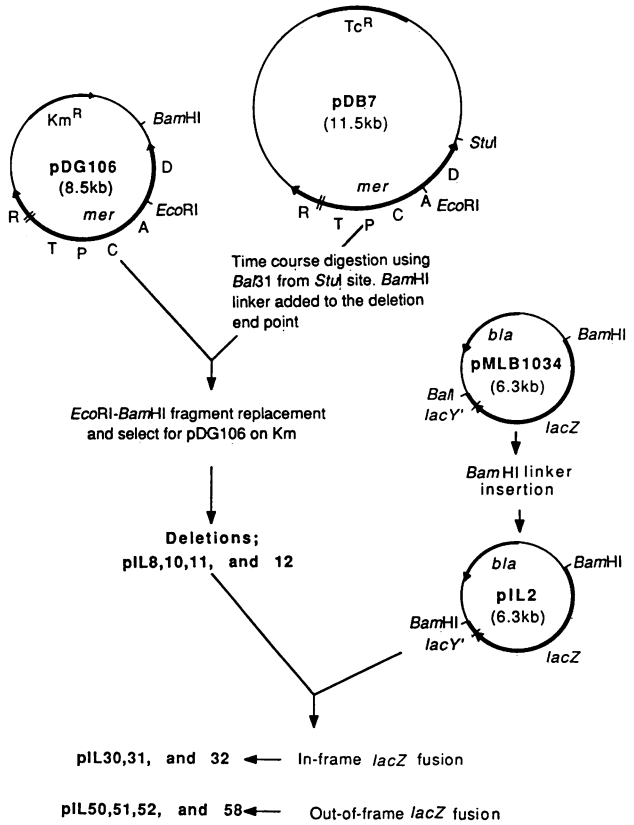

FIG. 2. Construction of *merD-lacZ* and *merA-lacZ* protein fusions. The *Eco*RI-*Stu*I fragment with a *Bam*HI linker added to the *Stu*I site was used to replace the *Eco*RI-*Bam*HI fragment of pDG106 to construct pIL8. For deletion endpoints of pIL10, pIL11, and pIL12, see Fig. 1. *Bam*HI linker was inserted in the *Bal*I site in pMLB1034 (24) to construct pIL2. Reading frames of fused genes were adjusted by using *Bgl*II linker. Km, Kanamycin; kb, kilobases.

What does *merD* do? As yet, we do not know whether the low level of *merD* expression is a result of the translational impediments suggested above or of the strong polarity observed in mRNA transcription (B. D. Gambill, Ph.D. thesis, University of Georgia, Athens, 1988). Densitometric analysis of *mer* protein expression in minicells (16) showed that of the four *mer* operon proteins detectable by this method, *merA* has 4- to 5-fold-lower expression than *merT* and *merC* and 10- to 20-fold-lower expression than the periplasmic protein *merP* (data not shown). Thus, since *merD* has 10- to 15-fold-lower expression than *merA*, it is not surprising that it was not detected in the minicell expression system. In addition, its similarity in size to the cotranscribed *merT* and *merC* gene products might have obscured the presence of *merD*. This low level of expression makes it less likely that *merD* has a role in known operon functions of Hg(II) transport (*merTP* and possibly *merC*) and enzymatic reduction of Hg(II) to Hg(0) (*merA*). Recently, the *merD* protein of Tn*501* has been detected by using a P_{tac} overexpression system (L. Shewchuk and C. Walsh, personal communication).

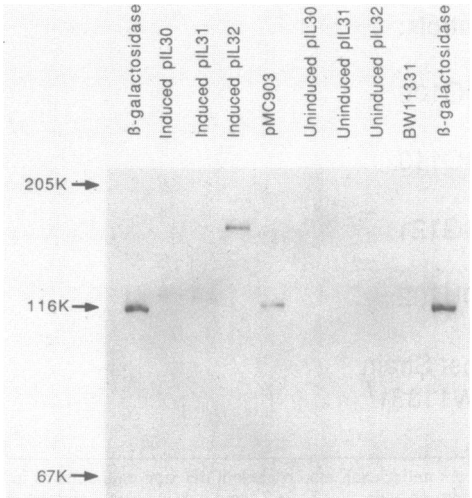

FIG. 3. Detection of MerD-LacZ fusion proteins by immunoblotting. Protein molecular weight standards (in thousands) are indicated with arrows. Authentic β-galactosidase and β-galactosidase from pMC903 (5) are used for blotting size standard. See text for details.

On the basis of its sequence similarity to *merR* and its apparent low abundance, the possibility that *merD* has a role, albeit ancillary, in regulation of the *mer* operon is worth considering. It is also possible that apart from encoding a protein, the *merD* mRNA secondary structure (which includes several strong potential stem-loop structures) has a role in *mer* message turnover (27). Our current genetic analysis of this system is directed towards resolving these questions.

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