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Down regulation of the mercury resistance operon by the most promoter-distal gene *merD*

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Summary. The effect of the *merD* gene on the expression of the *mer* operon was determined from the rates of accumulation of *merA-lacZ* fusion protein in the presence and absence of an active *merD* gene *in trans*. In the presence of the *merD* gene, β-galactosidase activity was 2- to 4-fold lower. The *merD* gene was cloned in a T7 promoter expression vector and the MerD protein product was visualized by autoradiography.

Key words: Mercury operon – *merD* regulation – *merR* regulation – Metal regulation – Operon regulation

Introduction

merD is an open reading frame of 366 nucleotides (363 in the Tn*21 mer* operon) in the structural gene cluster of the mercury resistance operons of transposons Tn*501* and Tn*21* (Brown et al. 1986) and plasmid pDU1358 (Griffin et al. 1987). Mutants in *merD* were first obtained by insertion of Tn*5* in the mercury resistance operon of Tn*21* (Ni'Bhriain et al. 1983), and later by deletion of part of the *merD* open reading frame in the mercury resistance operon of Tn*501* (Itoh and Haas 1985). In both cases, the resulting mutant operon gave a lower level of mercury resistance to the cell compared to the intact operon (Brown et al. 1986).

Gambill and Summers (1989) have recently detected a *merD-lacZ* protein fusion product. However, although there is a very strong DNA sequence homology among the three *merD* open reading frames (pDU1358 and Tn*501*, 91% identical nucleotides; pDU1358 and Tn*21*, 81%; Tn*501* and Tn*21*, 83%) which suggests function(s) for the *merD* gene product, no protein corresponding to the predicted MerD polypeptide has been identified in cells expressing the *mer* operon (Ni'Bhriain and Foster 1986). It is possible that the reduced *mer* operon function associated with *merD* mutations results from effects at the nucleic acid level (mRNA synthesis or stability; translational control) and not from effects of the translated *merD* protein product. We have investigated the possible role of the *merD* gene from the broad-spectrum *mer* operon of plasmid pDU1358 in the regulation of the *mer* operon. The effect of the *merD* gene was measured *in trans* as induction of the reporter gene product β-galactosidase produced from a *merA-lacZ* transcriptional fusion. The results of these experiments, reported in this paper, suggest that the *merD* gene product may have regulatory functions.

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Materials and methods

Bacterial strains and plasmid constructions. Escherichia coli strains used have been described elsewhere. Strain MC1061 (Miller 1987) was used as plasmid recipient in Hg^{2+} resistance measurements, strain K-38 containing plasmid pGP1-2 (Tabor and Richardson 1985) was used in protein expression experiments, and strain DU511 containing plasmid pDU1161 (Ni'Bhriain et al. 1983) was used in complementation assays.

The plasmids containing the *merD* reading frame in the orientation of the T7 promoter (pGN117-1) or in the opposite orientation (pGN117-2) were constructed by ligating a 1.1 kb *Nsi*I-*Pst*I fragment containing the entire reading frame of *merD* including 25 nucleotides upstream of the predicted *merD* ribosome binding site (Griffin et al. 1987) into the *Pst*I site of the polylinker region of plasmid pGEM3 (Promega Biotechnology, Madison, Wis). The construction of plasmids pGN120 and pGN107-2, used in the complementation assays, is outlined in Fig. 1. pGN120 was obtained by cloning the *Bgl*II-*Pst*I fragment of pHG103 (containing the entire broad-spectrum mercury resistance determinant; Griffin et al. 1987) into the *Sca*I site of pBR322 after polishing the ends of the fragment with Klenow DNA polymerase. Plasmid pGN107-2 was generated in two steps: first, a *merD* deleted operon was made by partial digestion of mGN103 (containing the *Bgl*II-*Pst*I fragment of pHG103 cloned in the M13 derivative mTM010; Misra 1987) with *Ava*II (which cuts in the *merB* gene of the operon) and complete digestion with *Pst*I (which has a single site in the 3′ distal polylinker region of the mTM010 vector). After polishing the ends with Klenow DNA polymerase, the linearized phage was ligated. The *merD* deleted *mer* determinant was then separated from the phage mTM010 vector by digestion with *Sal*I, which has recognition sites distal to *merR* (at the 5′ end of the operon) and in the phage polylinker region (3′ to the operon). After polishing the ends, the fragment was cloned into the *Sca*I site of pBR322.

In vivo expression of MerD. The T7 RNA polymerase expression system (Tabor and Richardson 1985) was used

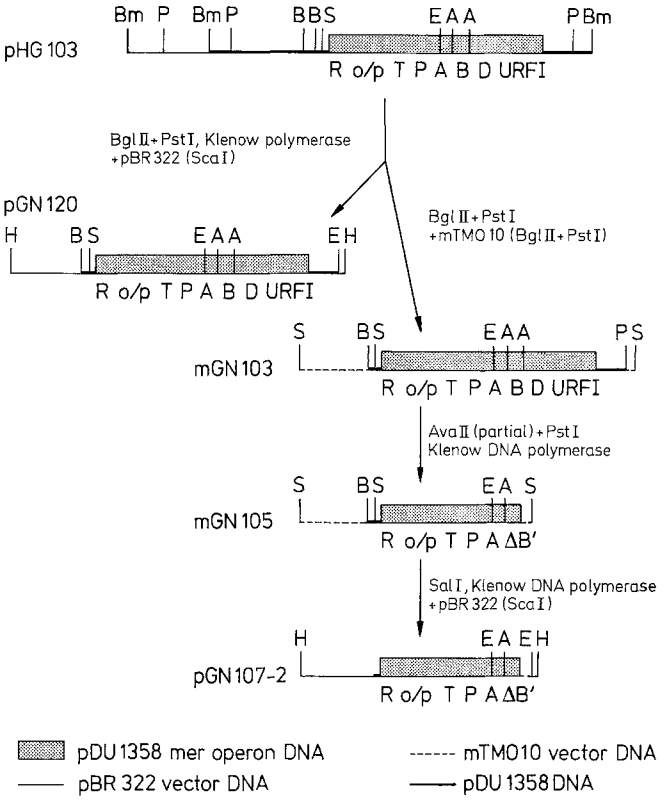

Fig. 1. Cloning of the constructs used: plasmid pHG103 (Griffin et al. 1987), shown linearized at a *Bam*HI site, was used as donor of *mer* DNA (shown *shaded*) which was then cloned into pBR322 (to give the recombinant plasmid pGN120, shown linearized at a *Hin*dIII site) and into mTM010 (to give the recombinant phage mGN103, shown *linearized* at a *Sal*I site). The *merD*$^-$ mutant pGN107-2 was obtained by partial digestion of mGN103 with *Ava*II, and insertion of the deleted fragment into pBR322. Restriction enzymes: Bm, *Bam*HI; P, *Pst*I; B, *Bgl*II; S, *Sal*I; E, *Eco*RI; H, *Hin*dIII; A, *Ava*II. *mer* operon genes: R, regulatory gene *merR*; T and P, transport genes *merT* and *merP*; A, mercuric reductase *merA*; B, lyase *merB*; D, *merD*; o/p, operator/promoter. Not drawn to scale

to identify the *merD* gene product. The system consists of a plasmid expressing the phage T7 RNA polymerase under the control of a temperature-sensitive lambda phage promoter P_L (pGP1-2) (Tabor and Richardson 1985) and a second vector, pGEM3 with the phage T7 promoter followed by a polylinker restriction endonuclease site. This system allows selective expression of cloned genes from the unique phage T7 promoter and inhibition of host cell RNA polymerase by rifampicin. *E. coli* cells strain K-38 (Tabor and Richardson 1985) containing both pGN117-1 and pGP1-2 or pGN117-2 and pGP1-2 were grown overnight at 30° C in Luria broth with 50 μg/ml each of ampicillin and kanamycin. The cultures were diluted 40-fold into fresh Luria broth, and incubated for 3 h with shaking at 30° C. Five-hundred microliters of the cell suspension was centrifuged, washed with 3 ml of M9 medium (containing in 1 l: 1 g NH_4Cl, 3 g KH_2PO_4, 6 g Na_2HPO_4, 1.4 g glucose, and 1 ml of 1 M $MgSO_4$), then suspended in 1.0 ml of M9 medium supplemented with 20 μg/ml thiamine and 0.01% 18 amino acids (minus cysteine and methionine) and allowed to grow at 30° C for 60 min. After heat induction of T7 RNA polymerase (42° C for 15 min), 200 μg/ml rifampicin (Sigma Biochemicals, St. Louis, MO) was added to inhibit cellular RNA polymerase, and incubation was continued at 42° C for 10 min. The temperature was shifted to 30° C for 20 min, and the cells were pulsed with 20 μCi of ^{35}S-methionine for 5 min, centrifuged, suspended in 120 μl denaturing buffer (50 mM TRIS-HCl, pH 6.7, containing in 1 l: 10 g sodium dodecylsulfate, 10 ml β-mercaptoethanol, 0.2 g bromophenol blue, 100 g sucrose, and 2 mM EDTA), and heated at 95° C for 3 min. Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecylsulfate (Davis et al. 1986). Autoradiography was performed using Kodak SB5 film (Eastman Kodak Company, Rochester, NY).

Susceptibility to mercurial compounds. Resistance to mercury was determined in liquid medium by monitoring growth by the turbidity of the culture after addition of Hg^{2+} (5 to 40 μM).

β-Galactosidase assays. The production of β-galactosidase from cells containing pDU1161 (Ni'Bhriain et al. 1983) was measured as described by Miller (1972).

Results

Visualization of the merD *product*

Figure 2 shows the autoradiogram of ^{35}S-methionine-labeled proteins from cells containing plasmids pGEM3, pGN117-1, and pGN117-2. Only pGN117-1 synthesized a protein of apparent molecular weight similar to that predicted for MerD (13.0 kDa; Fig. 2, lane 2). However, that protein was barely detectable; to visualize it, a long exposure was necessary (72 h) which resulted in a very high background. This may be related to the failure to detect MerD under *mer* promoter control (Ni'Bhriain and Foster 1986) and the expected low level of synthesis for a regulatory protein.

Phenotype of the merD$^-$ *deletion mutant of the mercury resistance operon of plasmid pDU1358*

Cells containing the deletion mutant plasmid pGN107-2 had a lower level of resistance to Hg^{2+} compared to those containing the intact operon (data not shown). These results are in agreement with similar work of Ni'Bhriain et al. (1983) and Brown et al. (1986), who found decreased resistance to Hg^{2+} in cells containing *merD* insertion or deletion mutants of the narrow-spectrum mercury resistance operons of R100 and Tn*501*.

Inducibility of the wild type and merD$^-$ *deletion mutant* mer *operons with inorganic and organo-mercuric ions*

The effect of the *merD* gene on the inducibility of the pDU1358 *mer* operon was studied in complementation assays between plasmid pGN120 (intact *mer* operon) or plasmid pGN107-2 (*merB*$^-$ *merD*$^-$ deletion mutant) and plasmid pDU1161, a derivative of plasmid R100 with an insertion inactivated *merR* gene and with a transcriptional *merA-lacZ* fusion. pDU1161 is also *merD*$^-$, since all nucleotides distal to the 5′ end of the *merA* gene were removed (Griffin et al. 1987; Ni'Bhriain et al. 1983). The complementation assays were extended to the narrow-spectrum mercury resistance determinant of plasmid R100, using the recombinant plasmid pDU1003, containing the entire narrow-spectrum

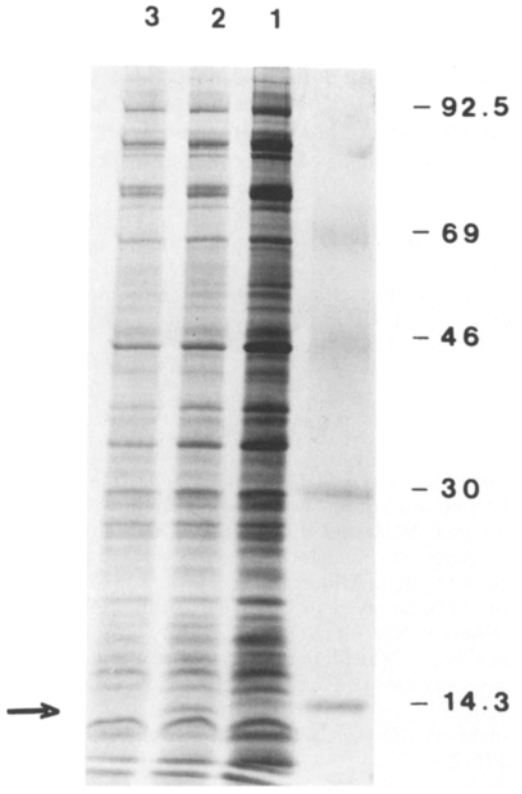

Fig. 2. Identification of the *merD* gene product under T7 RNA polymerase control. The 1.1 kb *Nsi*I-*Pst*I fragment containing the pDU1358 *merD* gene was cloned in the orientation of the T7 promoter (lane 2) and in the opposite orientation (lane 3). After heat induction, inhibition of host RNA polymerase with rifampicin, protein labeling with ^{35}S-methionine, solubilization and electrophoresis of cell proteins, and autoradiography, a polypeptide band was visible from expression of the cloned fragment in one orientation (lane 2), but not in the other orientation (lane 3) or with the vector alone (lane 1). The band marked with an *arrow* corresponds to a polypeptide of apparent molecular weight of 13.0 kDa.

mercury resistance of plasmid R100 (Ni'Bhriain et al. 1983), and a derivative of pDU1003, plasmid pDU1003-45, with a Tn*5* insertion in *merD* (Ni'Bhriain et al. 1983). If *merD* functions at the protein level, then its effect should be seen in *trans* complementation experiments, which can be most clearly carried out with *merA-lacZ* fusions in the absence of complicating *mer* enzyme activity. Using phenylmercuric acetate as inducer separated effects of broad-versus narrow-spectrum *merR* and possibly *merD* function. In the absence of MerD, the assays consistently showed that 2-fold more β-galactosidase was synthesized than in the presence of MerD (Fig. 3A). The narrow-spectrum MerR failed to induce with phenylmercuric acetate, with or without MerD (Fig. 3A). It is of interest in these experiments that the R100 MerD appears to function with the pDU1358 MerR in inhibiting β-galactosidase activity (Nucifora et al. 1989). With inorganic Hg^{2+} as inducer, both the R100 and the pDU1358 systems repeatedly showed 4-fold less β-galactosidase synthesis in the presence than in the absence of MerD (Fig. 3B).

Discussion

The existence and function of *merD* has been on a rather tentative basis (Brown et al. 1986; Ni'Bhriain et al. 1983). Our results add to the evidence for *merD* as a protein regulatory determinant. It is possible that the *merD* sequence functions at the level of transcriptional or translational control (in the synthesis, stability, or translation of the mRNA). The existence of inverted repeat sequences (Fig. 4) within *merD* raises this possibility. Deletion of these sequences in *merD*$^-$ mutants could contribute to the degradation of *mer* mRNA, resulting in the loss of net synthesis of mercuric reductase and organomercurial lyase in the cell. Under such conditions, higher levels of Hg^{2+} or organomercurial inducers would be available inside the cell to promote the activation of *merA-lacZ* transcription.

The increased activation with phenylmercuric acetate of the *mer* operon in *trans* complementation with pGN107-2 (Fig. 3A) suggests a diffusible product functions for *merD*. A contributing factor to the enhanced production of β-galactosidase with pGN107-2 might be an accumulation inside the cell of the phenylmercuric acetate inducer at higher levels compared to the cells expressing the complete (*merB*$^+$) operon. However, previous results with the hybrid pDU1358/R100 *mer* operon of pGN101, which as pGN107-2 is *merB*$^-$ but contains *merD* from R100, showed that the production of β-galactosidase was reduced compared to the complete *merB*$^+$ operon (Nucifora et al. 1989).

It seems more likely that the inhibition of the *mer* operon occurs due to the production of MerD protein (Fig. 2), which acts in a *trans* regulatory manner. The predicted MerD polypeptide sequence aligns with the amino acid sequence of the MerR, the *trans*-acting regulatory protein of gram-negative mercury resistance operons, with about 26% amino acid identity (Brown et al. 1986). The

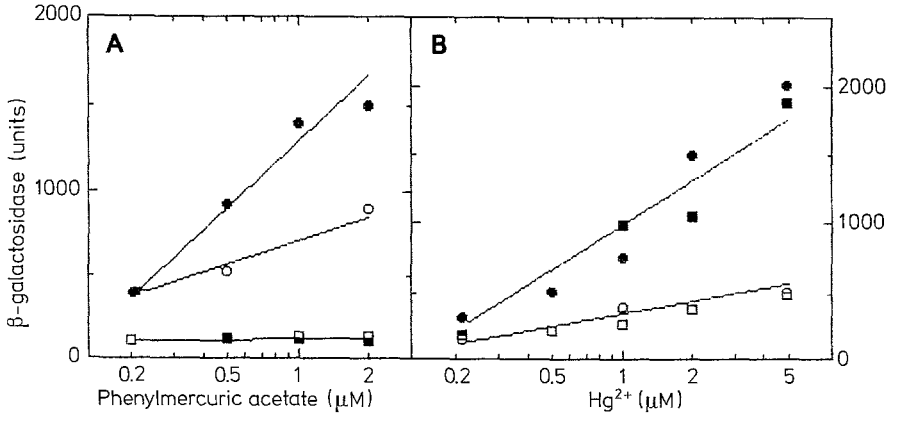

Fig. 3A and B. Inducibility of the *mer* operon measured as expression of *merA-lacZ* fusion with **A** phenylmercuric acetate or **B** Hg^{2+} as inducer. The complementing plasmids *in trans* were pGN120 (○), pGN107-2 (●), pDU1003 (□), and pDU1003-45 (■)

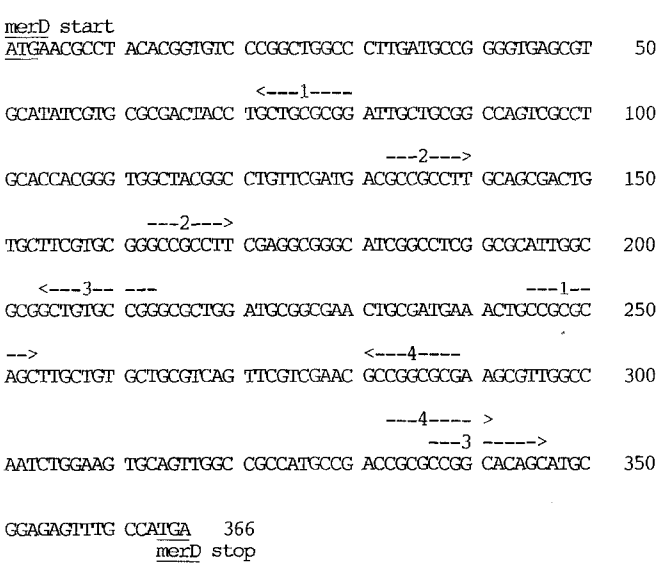

Fig. 4. Inverted (IR) and direct (DR) repeats within the *merD* open reading frame of plasmid pDU1358. Only the largest repeats (IRs of 9 and 10 nucleotides; DR of 8 nucleotides) are shown

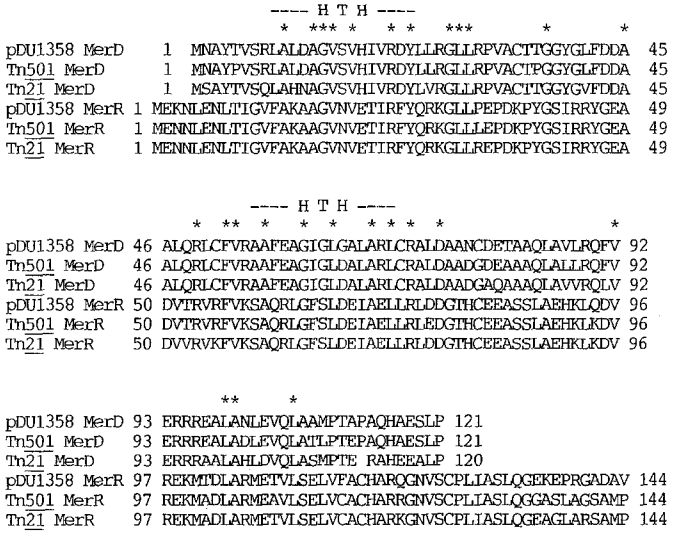

Fig. 5. Alignment of MerR and MerD protein sequences from gram-negative *mer* operons. The *asterisks* indicate identities among all six proteins. The two proposed helix-turn-helix (HTH) structures are indicated. Appropriate references are: Tn*501* MerD and R100 MerD, Brown et al. (1986); Tn*501* MerR and Tn*21* (≡R100) MerR, Misra et al. (1984); pDU1358 MerD, Griffin et al. (1987); pDU1358 MerR, Nucifora et al. (1989)

conservation of residues is highest in the region predicted to form the helix-turn-helix DNA binding structure of MerR (Fig. 5). The existence of the strong sequence homology between MerD and MerR at the polypeptide level suggests that the MerD polypeptide is synthesized and functions in a regulatory fashion. This was first pointed out by Brown et al. (1986) in their report of the nucleotide sequence and has subsequently been reviewed (Silver and Misra 1988; Summers 1986). The current experimental results, including direct demonstration of the production of the MerD polypeptide under T7 promoter control and the functioning of the *merD* system *in trans* in lowering expression in the *merA-lacZ* system suggests that *merD* may indeed function via a protein product. Nevertheless, in the absence of in vitro analysis of the interaction of the MerD protein with MerR and/or the operator/promoter nucleotide sequence, the mechanism of function of *merD* remains unclear.

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