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Effect of Gene Amplification on Mercuric Ion Reduction Activity of *Escherichia coli*

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The mercury resistance (*mer*) operon of plasmid R100 was cloned onto various plasmid vectors to study the effect of *mer* gene amplification on the rate of Hg^{2+} reduction by *Escherichia coli* cells. The plasmids were maintained at copy numbers ranging from 3 to 140 copies per cell. The overall Hg^{2+} reduction rate of intact cells increased only 2.4-fold for the 47-fold gene amplification. In contrast, the rate of the cytoplasmic reduction reaction, measured in permeabilized cells, increased linearly with increasing gene copy number, resulting in a 6.8-fold overall amplification. RNA hybridizations indicated that mRNA of the cytoplasmic mercuric reductase (*merA* gene product) increased 11-fold with the 47-fold gene amplification, while mRNA of the transport protein (*merT* gene product) increased only 5.4-fold. Radiolabeled proteins produced in maxicells were used to correlate the expression levels of the *mer* polypeptides with the measured reduction rates. The results indicated that, with increasing gene copy number, there was an approximately 5-fold increase in the *merA* gene product compared with a 2.5-fold increase in the *merT* gene product. These data demonstrate a parallel increase of Hg^{2+} reduction activity and transport protein expression in intact cells with plasmids with different copy numbers. In contrast, the expression level of the mercuric reductase gene underwent higher amplification than that of the transport genes at both the RNA and protein levels as plasmid copy number increased.

Reduction of mercuric ions (Hg^{2+}) to elemental mercury (Hg^0) in *Escherichia coli* is mediated by polypeptides of the plasmid-borne mercury resistance (*mer*) operon (19, 29). The mercury resistance determinant of plasmid R100 consists of six genes (*merRTPCAD*) (8, 15, 26, 30). Mercuric ion reduction is Hg^{2+} inducible (39). Negative and positive regulation of *mer* gene transcription is exerted by the *merR* gene product, which also negatively autoregulates its own transcription (15, 30). The *merD* gene product is thought to have a negative regulatory effect on the expression of the *mer* operon (31).

A model for mercury resistance has been proposed by Brown (7). The periplasmic *merP* and the inner membrane *merT* gene products are believed to transport Hg^{2+} across the cellular envelope into the cytoplasm (21, 38). The presence of those proteins is essential for Hg^{2+} reduction (21). An additional polypeptide, the *merC* gene product, is located in the inner membrane (29) and may also be involved in Hg^{2+} transport (38). In the cytoplasm, the flavin adenine dinucleotide-containing enzyme mercuric reductase (*merA* gene product; EC 1.16.1.1) catalyzes the reduction of Hg^{2+} to Hg^0 at the expense of NADPH (17, 37). The purified enzyme requires thiols or EDTA for catalytic activity (24, 34). Cysteyl residues of the polypeptides are believed to mediate the binding of Hg^{2+} (13, 26).

The proposed model for mercury resistance suggests that reduction of Hg^{2+} in the cells is carried out by the coordinated action of the transport proteins and mercuric reductase. We have previously measured the overall rate of Hg^{2+} reduction by intact *E. coli* cells and used ether-permeabilized cells to determine the rate of the enzymatic reaction (32). The overall rate was found to be several times lower than the reaction rate. This prompted us to investigate means to increase the reduction activity by intact cells. The mercuric ion reduction system was selected as representative of complex biochemical reactions that are carried out by the coordinated action of several proteins and enzymes in intact cells and are coupled to the cellular metabolism. Applications of complex biocatalysis include the production of amino acids, vitamins, cofactors, antibiotics, and chemicals. The ultimate goal of this investigation is to optimize the rate of such processes in order to advance the current status of applied biocatalysis.

Here we present the effect of *mer* gene amplification on the Hg^{2+} reduction rate of whole cells and the expression level of the *mer* operon. Gene amplification was achieved by cloning the *mer* operon of plasmid R100 in plasmid vectors maintained at copy numbers ranging from 3 to 140 per cell. Intact and ether-permeabilized cells harboring those recombinant plasmids were used to determine the effect of gene amplification on the Hg^{2+} transport and reduction reaction rates, respectively. Gene-specific hybridization to mRNA was used to quantitate the effect of gene dosage on mRNA levels. Furthermore, maxicells were employed to determine the effect of gene amplification on the expression levels of the *mer* polypeptides.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains C600r$^-$m$^+$ (3) and JM109 (44) were used as hosts for the *mer* operon plasmids constructed and employed in this study. *E. coli* MM294A (3) was used to prepare maxicells for labeling in order to measure the expression levels of the plasmid-encoded *mer* polypeptides.

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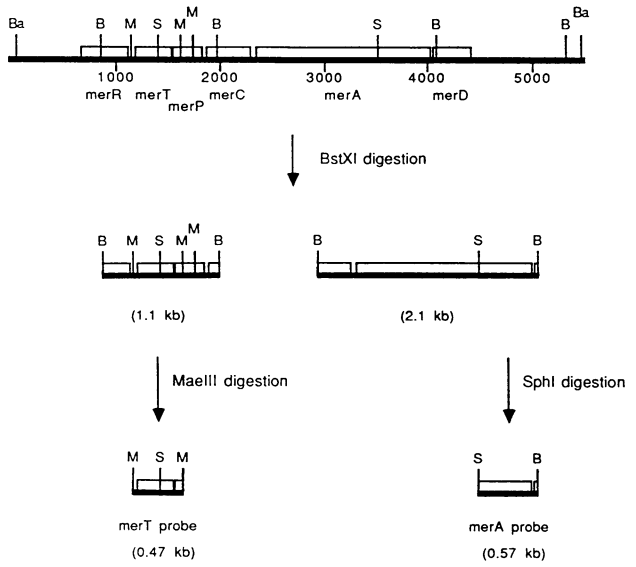

FIG. 1. Physical map of the *mer* operon. The 5.4-kb *Bal*I fragment was used to construct the various *mer* plasmids. The *merT* probe was prepared by digesting the 1.1-kb *Bst*XI fragment with *Mae*III. This fragment contains the entire *merT* gene and a 5′ proximal portion of *merP*. The *merA* probe was isolated by digesting the 2.1-kb *Bst*XI fragment with *Sph*I. This fragment contains a 3′ portion of the *merA* gene and a 5′ proximal portion of *merD*. Restriction endonuclease cleavage sites are labeled for *Bal*I (Ba), *Bst*XI (B), *Mae*III (M), and *Sph*I (S).

Plasmids R100 (23) and pDU1003 (30) were used as sources of the *mer* operon. Plasmids pBR322 (5), pACYC184 (12), and pUC19 (44) were the vectors with different copy numbers used for cloning the *mer* operon from pDU1003. Plasmid R100 is 90 kb in length and encodes resistance to chloramphenicol, tetracycline, streptomycin and spectinomycin, sulfonamides, fusidic acid, and Hg^{2+} (23). It is maintained at about one to two copies per genome (27). Plasmid pDU1003, originally constructed by cloning the *mer* operon from R100 into pBR322, is 11.3 kb in length, has a reported copy number of 30 per cell, and encodes resistance to tetracycline and Hg^{2+} (30). Plasmid pBR322 is 4.4 kb in length, contains resistance markers for ampicillin and tetracycline (5, 40), and has a reported copy number of 57 (11) or 67 (10) per cell. Plasmid pACYC184 is 4.2 kb in length (35) and encodes resistance to chloramphenicol and tetracycline (12). It is maintained at a copy number of 18 per genome (12). pUC19 is a 2.7-kb derivative of pBR322 that contains the *lac* promoter-operator, the *lacZ* α complementation sequence, and a polylinker sequence and encodes resistance to ampicillin (44). It has a reported copy number of 500 to 700 per cell (11).

Construction of the *mer* plasmids. For cloning the *mer* operon, plasmid pDU1003 was digested with *Bal*I, and the 5.4-kb DNA fragment containing the *mer* operon (Fig. 1) was isolated by electroelution from a 1% agarose gel (22). Plasmids pBR322, pACYC184, and pUC19 were linearized by restriction endonucleases *Eco*RV, *Eco*RI, and *Eco*O109, respectively. The 5′-protruding ends were filled in by the Klenow enzyme and ligated to the 5.4-kb *mer* fragment. The constructed plasmids were designated pBRmer, pACYCmer, and pUCmer, respectively. Prior to ligation, all linearized plasmids were dephosphorylated with calf intestine alkaline phosphatase to suppress recircularization of the vector.

Frozen competent $C600r^-m^+$ cells, prepared as described by Hanahan (16), were transformed by the ligation products, and transformants were selected with 10 μg of tetracycline per ml (for pACYCmer) or 50 μg of ampicillin per ml (for pBRmer and pUCmer). DNA was isolated from the selected colonies (22), and the construction of the recombinant plasmids was confirmed by restriction enzyme mapping analysis. Cells containing the *mer* constructs exhibited resistance to 10 μM $Hg(NO_3)_2$.

Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and Bethesda Research Laboratories (Gaithersburg, Md.). Klenow and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, Ind.).

Preparation of DNA hybridization probes. DNA probes for RNA analysis were prepared by subcloning regions of the *mer* operon (Fig. 1). A 0.47-kb *Mae*III fragment and a 0.57-kb *Bst*XI-*Sph*I fragment, isolated from the 5.4-kb *Bal*I fragment of pDU1003, were individually cloned into the *Sma*I-digested pGEM-3Z vector (Promega) and propagated in JM109 (44). The resulting plasmids, pGEM-T and pGEM-A, were used to detect *merT* and *merA* mRNA, respectively. The sizes and identities of the inserts were verified by restriction mapping. Purified *Eco*RI-*Hin*cII fragments of pGEM-T and pGEM-A were used as probes in the dot blot RNA hybridization experiments. Radiolabeled probes were prepared by the random priming reaction (14). The specific activities of probes A and T were 2.4×10^8 and 2.0×10^8 cpm/μg of DNA, respectively.

Mercuric ion reduction assay. Cells carrying the plasmid of interest were cultivated at 37°C in LB medium (22) containing 10 μM $Hg(NO_3)_2$ and 10 μg of tetracycline per ml (for R100, pDU1003, and pACYCmer) or 50 μg of ampicillin per ml (for pBRmer and pUCmer). The procedures for cell growth, induction with Hg^{2+}, and harvesting were carried out as described previously (32). Cell permeabilization was performed by a brief treatment with ethyl ether on ice (32, 43).

The reductase assay was carried out with 50 mM sodium phosphate buffer (pH 7.2) containing 200 μM Na_2EDTA, 1.0 mM β-mercaptoethanol, 200 μM $MgCl_2$, and 1.0 mM NADPH (32). Mercuric ions were added as a mixture of $Hg(NO_3)_2$ and $^{203}Hg(NO_3)_2$ (10.6 mCi/mg; Du Pont, Wilmington, Del.) at the desired concentration. The assay was initiated by the addition of cells, and the mixture was shaken at 200 rpm in a 37°C reciprocating water bath. The disappearance of $^{203}Hg^{2+}$ from the reaction buffer allowed monitoring of the reduction activity of the cells. Samples were removed periodically, and the amount of radioactivity was quantitated by liquid scintillation spectroscopy with a Beckman LS3801 scintillation counter. The data collected at intervals of 20 s during the first minute of the assay were used to calculate the initial Hg^{2+} reduction rate. Each assay was performed three times, each time in triplicate. The standard deviations of the measured initial reduction rates were less than 10%.

Protein concentration was determined by the method of Bradford (6), using γ-globulin as a standard. The final protein concentration in the assays was 0.25 to 0.29 mg/ml for the intact cells and 0.18 to 0.22 mg/ml for the permeabilized cells.

The initial reduction rates were normalized to the amount of total cellular protein and fitted to proposed models by nonlinear regression by using the Levenberg-Marquardt

TABLE 1. Kinetic parameters for Hg^{2+} reduction in intact and permeabilized cells harboring *mer* plasmid constructs of different copy numbers

Plasmid	Copy number[a] (copies cell)	$r_{max,I}$[b] (nmol of Hg^{2+} min^{-1} mg^{-1})	$K_{m,I}$[b] (μM)	$r_{max,P}$[c] (nmol of Hg^{2+} min^{-1} mg^{-1})	$K_{m,P}$[c] (μM)	$K_{i,P}$[c] (μM)
R100	3	8.2	3.8	45	12.6	96.7
pBRmer	67	13.4	4.7	152	15.9	91.5
pDU1003	78	17.5	3.6	168	22.8	87.4
pACYCmer	124	20.6	5.1	221	15.5	75.2
pUCmer	140	19.8	6.0	305	19.5	93.1

[a] The values represent the averages of four independent determinations, each carried out in triplicate. Standard deviation in all cases was less than 10%.
[b] The values of the parameters $r_{max,I}$ and $K_{m,I}$ of equation 1 for intact cells were determined from Fig. 2. The values represent the averages of three independent determinations, with standard deviations not exceeding 10%.
[c] The values of the parameters $r_{max,P}$, $K_{m,P}$, and $K_{i,P}$ of equation 2 for permeabilized cells were determined from Fig. 3. The values represent the averages of three independent determinations, with standard deviations of less than 15%.

least squares minimization algorithm (Microsoft Scientific Software, Salt Lake City, Utah).

Plasmid copy number determination. The copy number at which each plasmid was maintained in the host cells was determined by dot blot DNA hybridization (1). Cells suspended in sodium phosphate buffer (pH 7.2), which had been assayed for mercuric ion reduction activity, were sonicated, fixed onto a Zeta-Probe nylon membrane (Bio-Rad, Richmond, Calif.), and hybridized to the probe as described by the manufacturer's suggested hybridization protocol. The probe used was the 5.4-kb *Bal*I fragment from pDU1003 containing the *mer* operon, which had been ^{32}P labeled by the random priming method (14). This fragment contained DNA sequences common to all five plasmids. Known amounts of cesium chloride-purified pDU1003 plasmid DNA, ranging from 0.1 to 5 ng, were also hybridized to the probe to correlate the measured radioactivity level of the dots with the amount of plasmid DNA. The measured amounts of plasmid DNA were normalized to the protein concentration. The plasmid DNA content of the cells was expressed as the number of molecules of plasmid DNA per cell (copies per cell), assuming that 1 g of total cellular protein corresponds to 5×10^{12} cells and that each cell contains 2.45 genomes (1).

Protein identification in maxicells. Maxicells were used to specifically label plasmid-encoded polypeptides (36). The five plasmids R100, pBRmer, pDU1003, pACYCmer, and pUCmer were introduced into competent MM294A cells. Maxicells were prepared as described previously (36), except that irradiation was carried out with the short-wavelength light of a UVGL25 lamp for 6 min. For induction of the *mer* polypeptides, 10 μM $Hg(NO_3)_2$ was added to the cells during the 90-min period of labeling with 20 μCi of [^{35}S]methionine per ml (1,000 Ci/mmol; Amersham, Arlington Heights, Ill.).

Induced, uninduced, and plasmidless (MM294A) cells were harvested by centrifugation and resuspended in a denaturing solution containing 60 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 7.75% glycerol, and 0.025% bromphenol blue. After being heated for 5 min at 100°C, the samples were run on a 15% SDS-polyacrylamide gel (20). Rainbow markers (Amersham) were used as molecular weight standards. The gel was treated with Enhance (Du Pont), dried, and subjected to fluorography. The intensities of the polypeptide bands on the fluorogram were quantified by one-dimension video densitometry (model 620; Bio-Rad).

Isolation of total RNA. Cells were grown at 37°C in LB medium containing 10 μg of tetracycline per ml (for R100-containing cells) or 50 μg of ampicillin per ml (for pUCmer-containing cells). They were induced twice by the addition of 20 μM $Hg(NO_3)_2$ at an optical density at 650 nm (OD_{650}) of 0.1 and 0.2. Fresh culture was then inoculated with 5% of these induced cells and was again induced with 20 μM of $Hg(NO_3)_2$ at an OD_{650} of 0.1.

Cells were harvested at an OD_{650} of 0.3, chilled in ice with 2% sodium azide, pelleted by centrifugation, and lysed in 20 mM sodium acetate (pH 5.5)–0.5% SDS–1 mM EDTA. Total cellular RNA was recovered by extraction with hot phenol (2). An equal volume of hot phenol equilibrated with 20 mM sodium acetate (pH 4.9) was mixed with the lysed cells and shaken for 10 min at 60°C. The phases were separated by centrifugation, and the aqueous phase was extracted two more times. The RNA was precipitated from the aqueous phase by cold ethanol and redissolved in Tris-$MgCl_2$ buffer. After treatment with RNase-free DNase I (Promega) at 37°C for 60 min, the RNA was extracted with phenol-chloroform (1:1), precipitated with ethanol, and resuspended in 10 mM sodium phosphate (pH 7.0). Total RNA was quantified spectrophotometrically.

RNA dot blot hybridization. The total cellular RNA from both pUCmer- and R100-containing cells was denatured in 50% deionized formamide and 6% formaldehyde at 50°C for 1 h. Different amounts of denatured RNA, ranging from 0.2 to 3 μg of total RNA, were fixed to nylon membrane filters (GeneScreen Plus; Du Pont) and baked at 80°C under a vacuum for 1 h. The baked filters were prehybridized at 60°C for 15 min in the hybridization solution containing 1% SDS, 1 M sodium chloride, and 10% dextran sulfate. After addition of 1.1×10^7 cpm of probe DNA, the filters were hybridized at 60°C for 18 h.

The filters were washed twice for 5 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, twice for 30 min at 60°C with 2× SSC–1% SDS, twice for 30 min at 60°C with 0.1× SSC–1% SDS, and finally once with 0.1× SSC at room temperature. The filters were air dried. The radioactivity of each dot was quantified by using an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, Calif.).

RESULTS

Effect of gene amplification on the Hg^{2+} reduction rate. The copy numbers of the five plasmids R100, pBRmer, pDU1003, pACYCmer, and pUCmer in *E. coli* C600r$^-$m$^+$ cells were measured by dot blot DNA hybridization (Table 1). In order to correctly evaluate the effect of gene amplification on enzyme activity, it was essential to determine the actual

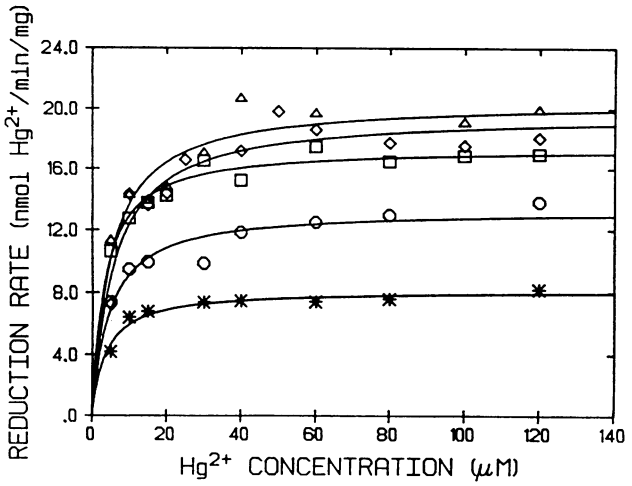

FIG. 2. Effect of gene amplification on the Hg^{2+} reduction rate of intact cells. C600 cells carrying *mer* plasmids of various copy numbers were assayed for reductase activity in the presence of 1 mM NADPH and various concentrations of Hg^{2+}. Symbols: *, R100; ○, pBRmer; □, pDU1003; △, pACYCmer; ◇, pUCmer.

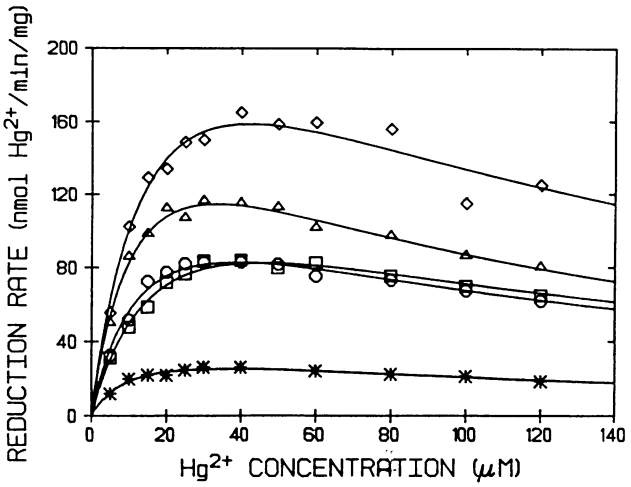

FIG. 3. Effect of gene amplification on the Hg^{2+} reduction rate of permeabilized cells. C600 cells carrying *mer* plasmids of various copy numbers were ether permeabilized and assayed for reductase activity in the presence of 1 mM NADPH and various concentrations of Hg^{2+}. Symbols: *, R100; ○, pBRmer; □, pDU1003; △, pACYCmer; ◇, pUCmer.

copy numbers of the *mer* plasmids in the same cells that were used to measure the Hg^{2+} reduction rate. Plasmid copy number determinations were carried out by using a fraction of the cells that were assayed for their reduction activities. The copy numbers of the various plasmids ranged from 3 to 140 plasmid copies per cell. This range of plasmid copy number represents a 47-fold increase in *mer* gene copy number when the R100 plasmid is compared with the pUCmer plasmid.

The reduction of Hg^{2+} involves the transport of Hg^{2+} across the cellular envelope and the reduction of Hg^{2+} to Hg^0 by mercuric reductase (7). Consequently, the overall reduction rate in intact cells is a function of both the Hg^{2+} transport and reduction rates. Since the transport process appears to be the rate-determining step, the overall reduction rate of intact cells can be used as a measurement of the Hg^{2+} transport rate (32). We have previously shown that the Hg^{2+} reduction rate of ether-permeabilized cells represents the catalytic rate of mercuric reductase (32). Therefore, intact and permeabilized cells can serve as a means to study the effect of gene amplification on the transport and reduction rates, respectively.

The Hg^{2+} reduction rate of intact cells harboring the *mer* plasmids was measured by using reaction buffer with Hg^{2+} concentrations ranging from 5 to 120 μM and with 1 mM NADPH. The time course of the residual Hg^{2+} concentration in the assay buffer was monitored, and the initial rate of Hg^{2+} reduction at each Hg^{2+} concentration was determined. For all cells, increasing Hg^{2+} concentration resulted initially in an increase of the reduction rate until it reached a plateau (Fig. 2). We have previously shown that the reduction rate, r_I, of intact cells is adequately described by a Michaelis-Menten equation (33), where $r_{max,I}$ is the maximal reduction rate and $K_{m,I}$ is the Michaelis constant for intact cells:

$$r_I = \frac{r_{max,I}[Hg^{2+}]}{K_{m,I} + [Hg^{2+}]} \quad (1)$$

Nonlinear regression was applied to fit equation 1 to these reaction rates. Within a statistical confidence range of 95%, the correlation coefficient was greater than 0.92 for all measurements. The values of $r_{max,I}$ and $K_{m,I}$ are summarized in Table 1.

The initial Hg^{2+} reduction rate in permeabilized cells carrying the five *mer* plasmids was also assayed in the presence of 5 to 120 μM Hg^{2+} (Fig. 3). The reduction rate initially increased with increasing Hg^{2+} concentration, and then the rate decreased at concentrations greater than approximately 40 μM. We have previously shown that the reduction rate, r_P, of permeabilized cells exhibits a substrate inhibition type of kinetics, described by equation 2:

$$r_P = \frac{r_{max,P}[Hg^{2+}]}{K_{m,P} + [Hg^{2+}] + [Hg^{2+}]^2/K_{i,P}} \quad (2)$$

where $r_{max,P}$ is the maximal reduction rate, $K_{m,P}$ is the Michaelis constant, and $K_{i,P}$ is the inhibition constant for permeabilized cells (33). By using the nonlinear regression algorithm, equation 2 was fitted to the permeabilized cell rates. At a statistical confidence level of 95%, the correlation coefficient was greater than 0.97 for all determinations. The values of $r_{max,P}$, $K_{m,P}$, and $K_{i,P}$ for each plasmid-containing cell type are shown in Table 1.

The determined maximal reduction rates of intact cells ($r_{max,I}$) and permeabilized cells ($r_{max,P}$) were plotted against the copy numbers of the respective plasmids (Fig. 4). There was an initial increase in the $r_{max,I}$ value as the gene copy number increased from 3 ($r_{max,I}$ = 8.2) to 67 ($r_{max,I}$ = 13.4) to 78 ($r_{max,I}$ = 17.5), but further increases in gene copy number did not significantly increase the $r_{max,I}$. With the 47-fold increase in gene copy number, there was an overall 2.4-fold increase in $r_{max,I}$. In contrast, the maximal reduction rates of permeabilized cells ($r_{max,P}$) demonstrated a linear relationship with gene copy number (Fig. 4), with a correlation coefficient of 0.98 within a confidence range of 95% and a standard deviation of 23 nmol of Hg^{2+} per min per mg. The overall 47-fold amplification of the gene copy number resulted in a 6.8-fold increase of the maximal rate of reduction by permeabilized cells. If one examines only the cases of pBRmer, pDU1003, pACYCmer, and pUCmer,

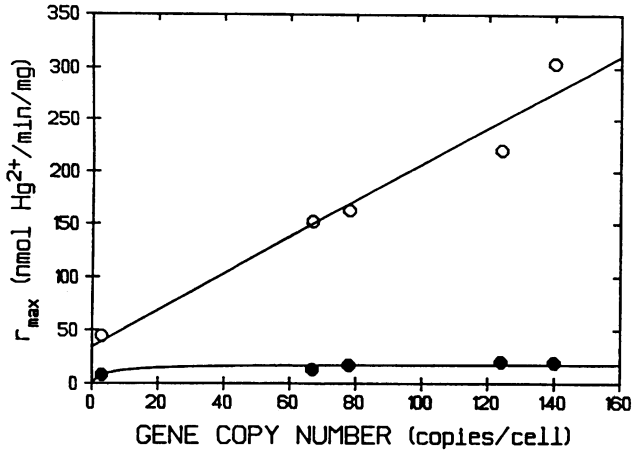

FIG. 4. Dependence of the maximal Hg^{2+} reduction rate on the copy number of the *mer* operon. The maximal reduction rates of intact ($r_{max,I}$) and permeabilized ($r_{max,P}$) cells containing *mer* plasmids of various copy numbers were obtained from the data presented in Table 1. Symbols: ●, intact cells; ○, permeabilized cells.

TABLE 2. Effect of *mer* gene amplification on mRNA and polypeptide synthesis

Plasmid	mRNA (relative hybridization/μg of RNA)[a]		Polypeptide (band intensity/mg of protein)[b]	
	merT	*merA*	*merT*	*merA*
R100	1.6	1.0	0.69	0.40
pBRmer	ND[c]	ND	0.97	1.05
pDU1003	ND	ND	1.04	1.46
pACYCmer	ND	ND	1.52	1.73
pUCmer	8.6	10.8	1.73	1.98

[a] RNA corresponding to each *mer* cistron was quantitated by dot blot hybridization of RNA to gene-specific probes and normalization of the numbers to the amount of total cellular RNA in the sample. The numbers are expressed relative to *merA* hybridization to RNA from the R100-containing cells. These values are the averages of 11 hybridization replicas, with less than 10% variability.

[b] The intensity of each band is expressed, in arbitrary units as determined by the densitometer, as the area underneath the peak corresponding to the band. That value was normalized to the total amount of protein of the respective sample.

[c] ND, not determined.

then $r_{max,P}$ appears to be proportional to the gene copy number; for a gene amplification of 2.1, from 67 copies per cell (pBRmer) to 140 copies per cell (pUCmer), the maximal reduction rate was amplified twofold.

The variation in gene copy number did not affect the values of $K_{m,I}$, $K_{m,P}$, and $K_{i,P}$, which fell within a narrow range around 4.6, 17, and 90 μM, respectively, for cells carrying the five *mer* plasmids (Table 1). Such consistency underlines the common identity of the *mer* transport proteins and mercuric reductase in all the plasmid-harboring cells, since $K_{m,I}$, $K_{m,P}$, and $K_{i,P}$ are intrinsic parameters of the polypeptides and therefore independent of their concentrations in the cell.

Effect of gene amplification on synthesis of *mer*-specific mRNAs. The relative amounts of *merT* and *merA* mRNAs transcribed from *mer* plasmids with the lowest (R100) and highest (pUCmer) copy numbers were determined by RNA dot blot hybridizations (Table 2). *merT*-specific mRNA increased about 5.4-fold with the 47-fold increase in plasmid copy number. When *merA*-specific mRNA was probed, an approximately 11-fold increase in transcript was observed with the 47-fold increase in gene dosage. These results indicated that the amplification of transcript levels did not parallel the increase in gene dosage for either the reductase or transport protein genes. However, the relative increase in *merT* mRNA was about twofold less than the increase in *merA* mRNA when transcripts synthesized in the R100- and pUCmer-containing cells were compared.

Effect of gene amplification on synthesis of the *mer* polypeptides. The relative concentrations of the proteins encoded by the *merT*, *merC*, *merP*, and *merA* genes were measured by SDS-polyacrylamide gel electrophoresis (20) of proteins produced in maxicells (*E. coli* MM294A) harboring the *mer* plasmids (Fig. 5). The use of maxicells has been previously proposed as a means to label and identify plasmid-encoded proteins, with little background from chromosome-encoded polypeptides (36).

Plasmidless cells expressed no detectable polypeptides, indicating little synthesis of chromosome-encoded polypeptides in the maxicells (Fig. 5, lane 1). Four inducible polypeptides were identified by comparing the induced (lanes 2 to 6) and uninduced (lanes 7 to 11) samples. The molecular masses of the four polypeptides were calculated to be 62, 17, 15.5, and 12 kDa, corresponding to the *merA*, *merT*, *merC*, and *merP* gene products. These sizes are in reasonable agreement with the 58.9-, 12.5-, 15-, and 9.5-kDa sizes that are predicted from the amino acid sequences of the polypeptides (4, 25, 26). The final size of the *merP* gene product is believed to be 7.5 kDa after the removal of a leader sequence (7, 25). However, its size has been found to be 12 kDa from a minicell study (18), identical to our determination. Although the predicted size of the *merT* gene product (12.5

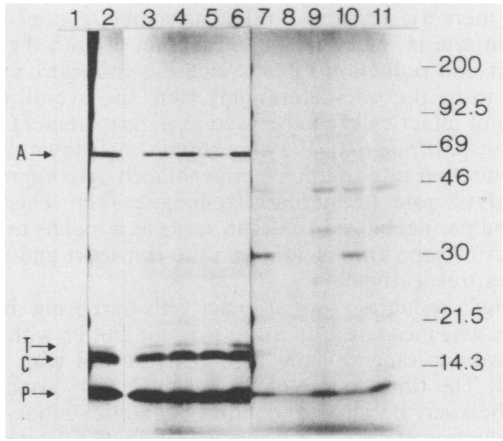

FIG. 5. Fluorogram of the SDS-polyacrylamide gel used for detecting the *mer* polypeptides synthesized in maxicells. Lane 1 contains a plasmidless sample (MM294A). Lanes 2 to 6 contain induced samples (lane 2, pDU1003; lane 3, pBRmer; lane 4, pACYCmer; lane 5, R100; lane 6, pUCmer). Lanes 7 to 11 contain uninduced samples (lane 7, pUCmer; lane 8, R100; lane 9, pACYCmer; lane 10, pBRmer; lane 11, pDU1003). Lanes 7 to 11 were loaded with about 2.5 times more total protein than lanes 1 to 6 and were taken from a darker exposure of the same gel to facilitate visualization of the polypeptides in uninduced cells. Molecular mass markers (in kilodaltons) are indicated at right. The sizes of the labeled polypeptides were as follows: A, 62 kDa (*merA* gene product); T, 17 kDa (*merT* gene product); C, 15.5 kDa (*merC* gene product); and P, 12 kDa (*merP* gene product).

kDa) is smaller than that of the *merC* gene product (15 kDa), it migrated more slowly. The same phenomenon has been reported previously and may be due to the hydrophobic nature of the *merT* gene product (29).

The intensities of the fluorogram bands were determined by one-dimension densitometry and were used as an index of the relative expression levels of the corresponding polypeptides in *E. coli* cells (Table 2). Over a 47-fold increase in gene copy number, a 2.5-fold amplification of the transport protein (*merT* gene product) and a 5-fold amplification of mercuric reductase (*merA* gene product) were observed. The increases in the relative concentrations of the transport protein and mercuric reductase, as a result of gene amplification, appeared to parallel the increases in the maximal rate of Hg^{2+} transport ($r_{max,I}$) and the maximal rate of the reduction reaction ($r_{max,P}$), respectively (Table 1). The amplification of the *merC* and *merP* gene products was similar to the amplification of the *merT* polypeptide (data not shown).

DISCUSSION

The maximal mercuric ion reduction rate measured in permeabilized cells ($r_{max,P}$) increased linearly with gene copy number. There was a 6.8-fold increase in rate for an overall 47-fold gene amplification (Table 1). Proportional increases in enzyme activity with gene copy number have also been observed for R1-encoded β-lactamase, chloramphenicol acetyltransferase, and streptomycin adenylase (42), as well as for the mercuric ion reduction activity of cell extracts from *E. coli* harboring R100 and its copy number variants (28). In contrast, the maximal transport rate ($r_{max,I}$), measured with intact cells, increased only 2.4 times with the 47-fold gene amplification (Table 1). A lack of proportionality between gene copy number and the intact-cell Hg^{2+} reduction rate has also been reported for *E. coli* cells harboring R100 and its derivatives (28). According to that study, an approximately 8-fold increase in plasmid copy number resulted in only a 1.5-fold increase of the intact-cell reduction rate. Qualitatively, the same phenomenon has been observed with the resistance levels to chloramphenicol and streptomycin of intact *E. coli* cells harboring plasmid R1 and copy number derivatives of this plasmid (42).

The observed limited increases in $r_{max,I}$ with gene copy number may be due to saturation of the membrane capacity with respect to the amount of transport proteins (*merT* and *merC* gene products) that can be associated with the membrane. Mercuric reductase is located in the cytoplasm, whereas the transport proteins function in the inner membrane. Various cytoplasmic enzymes and proteins have been amplified to levels representing 25 to 60% of the total protein content of the cell (9, 10). In contrast, membrane proteins have been amplified to much smaller extents, which may be attributed to the limited capacity of the inner membrane. When the *lacY* gene, which encodes lactose permease, was cloned in a pBR322 derivative, the protein was amplified to 12 to 16% of the total membrane protein content, which corresponds to only 1 to 3% of the total cellular protein (41). Alternatively, the limited increases in $r_{max,I}$ may result from energy limitations of the transport process, since it is speculated to be energy dependent (38).

The observed saturation of $r_{max,I}$ with increasing *mer* gene copy number may also be reflective of limited transport protein gene expression. Polypeptide synthesis in maxicells (Fig. 5) suggested that the small increase of $r_{max,I}$ is related to the limited amplification of transport proteins. Measurements of *mer*-specific mRNA also indicated that *merT* mRNA was amplified to a lesser extent than the *merA* mRNA, which could affect production of the corresponding polypeptides. Thus, the differentiation in the effect of gene amplification on $r_{max,P}$ and $r_{max,I}$ may be the result of the unequal amplification of mercuric reductase compared with that of the transport proteins (Table 2 and Fig. 5).

This experimental study suggests that the overall mercuric ion reduction rate of intact cells has limited potential for amplification with increasing gene copy number of the intact *mer* operon. An increase in the reduction activity of the intact cells would require an increase in the transport protein concentration. If the ratio of transport protein concentration to mercuric reductase concentration could be increased, the intracellular Hg^{2+} concentration might reach higher levels because of higher rates of Hg^{2+} transport. A higher intracellular Hg^{2+} concentration, in turn, would allow the reductase to function at a higher turnover rate, resulting in an overall higher rate of mercuric ion reduction activity of intact cells. However, the level of transport protein is maintained at a fairly constant concentration in spite of gene dosage, and it represents the major limitation in attempts to enhance the Hg^{2+} reduction activity in intact cells.

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