JOURNAL OF BACTERIOLOGY, Apr. 1979, p. 284-287
0021-9193/79/04-0284/04$02.00/0

Vol. 138, No. 1

Gene Copy Number Effects in the *mer* Operon of Plasmid NR1

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Received for publication 25 September 1978

The level of resistance to Hg^{2+} determined by the inducible *mer* operon of plasmid NR1 was essentially the same for three gene copy number variants in *Escherichia coli*, less in *Proteus mirabilis*, and intermediate in *P. mirabilis* "transitioned" to a high r-determinant gene copy number. Cell-free volatilization rates of radioactive mercury indicated increasing levels of intracellular mercuric reductase enzyme from low- to high-gene copy number forms in *P. mirabilis* and from low- to high-copy number forms in *E. coli*, but the additional enzyme in *E. coli* was effectively cryptic.

For both chromosomal (3) and plasmid genes (5, 14), increasing copies per cell generally leads to an increase in the protein gene product as well. We were therefore surprised to find no greater resistance to mercuric ions in cells carrying high copy number variants of the mercuric resistance determinants of plasmid NR1 (also known as R100 or R222). This note contains an explanation for this lack of gene copy number effect.

The bacterial strains and plasmids used in this study have been previously described (2, 4, 5). Exponentially growing cells contain three to four times as many plasmid copies of the round-of-replication mutant, pRR12, as of the wild-type NR1 plasmid (5). The small high-copy number plasmid ColE1 with the *mer* region from NR1 cloned into it (pRR130) is estimated to contain about 5 to 8 times the number of copies per cell as NR1 (4; unpublished data). The *Escherichia coli* K-12 sublines used in this work (CR34A and K245) do not appear to differ in gene copy number effects on the *mer* operon. "Transitioning" NR1 in *Proteus mirabilis* to a complex mixture of multimeric forms of the r-determinant was accomplished by at least two sequential rounds of growth in medium containing 100 μg of chloramphenicol per ml (2, 7).

Measuring the growth inhibition zones on petri dishes around paper disks containing Hg^{2+} showed no consistent effects of gene copy number with the three NR1 variants in *E. coli* (Table 1). *P. mirabilis* strain φS38 with plasmid NR1 showed greater sensitivity to Hg^{2+} than did the comparable *E. coli* strain. Strain φS38(NR1) was, however, more resistant than plasmidless strain φS38 and, after sequential growth on chloramphenicol to transition the plasmid to high r-determinant gene copy number (2, 7), the Hg^{2+} resistance level of *P. mirabilis* φS38(NR1) increased to a level near that of *E. coli*(NR1) (Table 1). Disks containing 10 to 100 nmol of Hg^{2+} and analogous experiments on the inhibition of growth by Hg^{2+} in liquid media also showed no difference in resistance levels between the resistant *E. coli* variants (data not shown).

Plasmid-determined mercuric resistance is due to the volatilization of mercury from the growth medium (9, 11, 15). The rates of volatilization of mercury from Hg^{2+} by intact washed cells closely paralleled the resistance levels (Table 1 and Fig. 1 and 2). The three *E. coli* strains showed relatively little difference in volatilization rates, and *P. mirabilis* φS38(NR1) volatilized mercury at lower rates than did comparable *E. coli* strains. Plasmidless strains did not cause mercury loss at measurable rates (Table 1).

One notable aspect of the detailed whole cell volatilization assays shown in Fig. 1 was the partial inhibition of mercury volatilization by the resistant cells by high substrate, i.e., by Hg^{2+} at levels comparable to those that cause growth inhibition. Such substrate inhibition has not been seen under comparable conditions with cell-free enzyme up to concentrations of 50 μM Hg^{2+} (8). The cell-free preparations demonstrated simple Michaelis-Menten kinetics with a K_m of 8 to 10 μM Hg^{2+} for all strains tested and with increasing V_{max}'s with gene copy number, as if increasing gene copies led to synthesis of increasing amounts of the mercuric reductase enzyme (Fig. 1). The *P. mirabilis* strain, in both low- and high-copy number form, showed less cell-free activity than did the three *E. coli* strains (Table 1).

TABLE 1. *Comparison of Hg^{2+} sensitivity[a] and enzyme activity levels[b]*

Strain	Inhibition zone (mm)	Mercuric reductase: Cells (V at 15 μM)	Mercuric reductase: Enzyme (V_{max})	Chloramphenicol acetyltransferase: enzyme (V_{max})
P. mirabilis				
φS38	7-9	<0.001	<0.001	<0.01
φS38(NR1)	4	0.006	0.009	0.29
Transitioned φS38(NR1)	2	0.015	0.033	2.32
E. coli: plasmidless strains	7-9	<0.001	<0.001	<0.01
CR34A(NR1)	0-2	0.037	0.062	0.87
CR34A(pRR12)	0-2	0.055	0.30	7.13
KP245(pRR130)	0-2	0.055	0.50	<0.01[c]

[a] Inhibition zone diameter with 50 nmol of Hg^{2+} per disk (range in different experiments) was as described earlier (16).

[b] Cells were grown in tryptone broth (8) and induced with 5 μM Hg^{2+}. After harvesting, whole cells and cell-free French press supernatant fluids (5,000 × *g*; 15 min) were assayed for mercury volatilization from 5 to 45 μM $^{203}Hg^{2+}$ (see Fig. 1 and 2). The V_{max}'s were determined from Lineweaver-Burk plots. The chloramphenicol acetyl-transferase assays were carried out with 0.1 mM acetyl-coenzyme A and 0.1 mM chloramphenicol, as described earlier (10). This is equivalent to determining the V_{max}. All enzyme activities are in units of μmol min^{-1} mg^{-1} of protein at 37°C.

[c] pRR130 does not contain a gene for this enzyme.

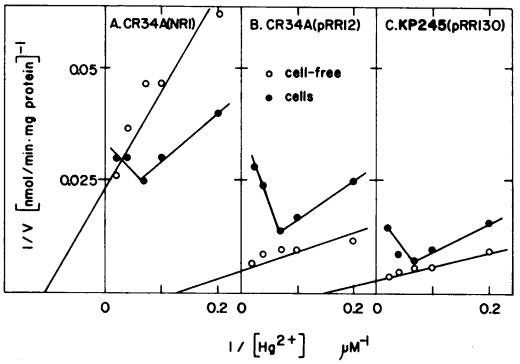

FIG. 1. *Comparison of cell and cell-free enzyme in E. coli. Washed cells (●) or cell-free low-speed supernatant fluids (○) were assayed for the initial rate of mercury volatilization with 5 to 45 μM $^{203}Hg^{2+}$ under conditions described earlier (8, 9).*

Careful, side-by-side, comparison of intact cell and cell-free enzyme volatilization rates (Fig. 1 and 2) has provided an explanation for these results of differing cell-free enzyme levels without differing intact cell volatilization rates. The whole cell level volatilization rates accounted for the cellular resistance level to Hg^{2+} and the intracellular mercuric reductase level (i.e., V_{max} for cell-free enzyme) showed an effect of gene copy number. With *P. mirabilis*, the intact cells at all concentrations below 45 μM showed higher volatilization rates than did the cell-free preparations obtained from them (Fig. 2). With both the nontransitioned and the transitioned *P. mirabilis* φS38(NR1), the cells appeared to concentrate the available substrate to a limited amount of intracellular enzyme (8, 13).

The situation was somewhat different with the gene copy number variants of NR1 *mer* in *E. coli*. The whole cell volatilization rates showed little (Fig. 1) or no (other experiments) difference between the three plasmids. Cell-free volatilization rates showed the expected effect of gene copy number. Whereas whole cell volatilization rates with *E. coli*(NR1) were generally more rapid at low substrate levels than comparable cell-free rates (Fig. 1A), with the high gene copy number variants the whole cell rates of mercury volatilization were always appreciably lower than with the cell-free enzyme preparations (Fig. 1B and C). These results are summarized in Table 1 along with control assays for chloramphenicol acetyltransferase, the enzyme determining chloramphenicol resistance that is governed by another gene on the r-determinant of NR1 and that is synthesized constitutively (4, 5, 14). The cell-free activities for both enzymes showed gene copy number effects, although the ratio of enzyme activities (high/low) were not the same for the two enzymes.

Rupturing cells in a French pressure cell was not the sole way of exposing the cryptic enzyme

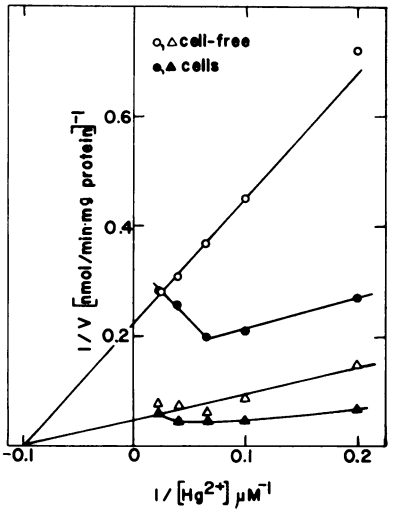

FIG. 2. *Comparison of cells and cell-free enzyme in P. mirabilis. Washed cells or cell-free low-speed centrifugal supernatant fluids of strain φS38(NR1) (●, ○) or transitioned φS38(NR1) (▲, △) were assayed for the initial rate of mercury volatilization.*

of high gene copy *E. coli* strains. When cells of strain KP245(pRR130) were exposed to 1% (vol/vol) toluene or to 1% (wt/vol) sodium deoxycholate for 10 min and subsequently assayed (after 40-fold dilution), the rate of mercury volatilization at all tested concentrations (5 to 45 μM Hg^{2+}) was higher than with intact cells, and the inhibition by high substrate seen with whole cells was not seen with toluene-treated cells (data not shown).

The model in Fig. 3 rationalizes the results in this report and our understanding of the effects of gene copy number on the functioning of the *mer* system of plasmid NR1 in *P. mirabilis* and in *E. coli*. Two components of the system are involved: the intracellular mercuric reductase (8) and a membrane transport carrier that brings the extracellular toxic substrate to the intracellular enzyme. Although this work on gene-dosage effects provides no direct evidence for a Hg^{2+} uptake system and that is only a plausible hypothesis at this stage, recent experiments with mutants and cloned fragments lacking the reductase volatilization activity have provided direct data on Hg^{2+} uptake by a Hg^{2+}-inducible plasmid-governed system (Nakahara et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H32, p. 109; H. Nakahara et al., submitted to J. Bacteriol.; T. J. Foster et al., submitted to J. Bacteriol.). The intracellular location for the enzyme (8, 13) may be essential for an enzyme requiring a small high-energy cofactor (NADPH). However, free intracellular Hg^{2+} would be rapidly bound to available sites of toxicity. These intracellular sites for Hg^{2+} are multiple and not pertinent to the experiments in this paper. However, a close association of the uptake sites to intracellular enzyme (as diagrammed in Fig. 3) would afford both access to the enzyme and protection for the cells. We have, however, found no evidence for a physical association of the enzyme with the cell surface; the mercuric reductase behaves in broken cell preparations as a soluble enzyme (8, 13; unpublished data).

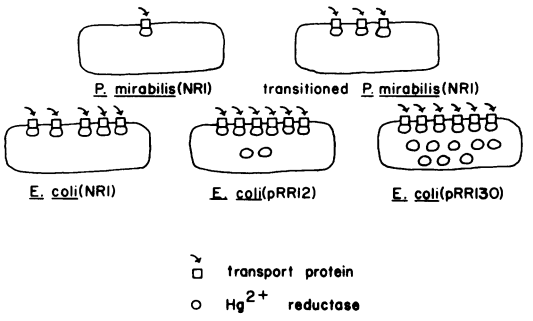

FIG. 3. *Model for gene copy number effects on mer operon functions.*

The model in Fig. 3 shows *P. mirabilis* ϕS38(NR1) with the lowest level of uptake function and of reductase enzyme of the five strains studied. With this strain, the uptake function effectively concentrates the Hg^{2+} and allows a higher volatilization rate for the intact cells than found with cell-free preparations. Transitioned ϕS38(NR1), with high gene copy number, showed more activity both in vivo and in vitro and greater Hg^{2+} resistance. *E. coli* strain CR34A(NR1) showed greater resistance and greater in vivo and in vitro volatilization rates (Table 1) than did even the high copy number transitioned *P. mirabilis*(NR1) (Fig. 3). With *E. coli* CR34A(NR1), the whole cell volatilization rate was greater than with cell-free preparations (Fig. 1). In other experiments, the balance was closer, and intact cells and cell-free preparations volatilized 5 μM Hg^{2+} at essentially identical rates (13). With the copy number mutant pRR12, the higher gene copy number did not result in appreciably higher intact cell resistance or volatilization rates, and we hypothesize that the number of possible cellular membrane sites for the transport function was saturated with strain CR34A(NR1) (Fig.3). The excess enzyme synthesized by strains CR34A(pRR12) and KP245(pRR130) was effectively "cryptic" and could be exposed by rupture of the cells.

With another plasmid (that lacks the mercuric resistance determinant), Uhlin and Nordström (14) showed linear relationships between gene copy number and cell-free enzyme rates for three antibiotic inactivating enzymes, but a lack of such an effect on resistance to chloramphenicol or streptomycin above a relative copy number of 2 to 3 times the wild-type plasmid level. These elegant studies involved a series of 12 plasmid mutants with variable plasmid copy number. Inactivation of chloramphenicol and streptomycin both require high-energy, normally intracellular cofactors (acetyl-coenzyme A and ATP, respectively). Resistance to ampicillin, mediated by β-lactamase without the need for a cofactor, increased directly with gene dosage in these studies (14). Uhlin and Nordström (14) discussed alternative explanations for this difference. Permeability effects have also been suggested to explain the lack of effect of gene copy number on resistance to aminoglycosides, when the genes for aminoglycoside adenylylating or acetylating enzymes were cloned into high-copy number plasmids (1).

This work had its impetus from the continuing correspondence and exchange of strains between laboratories concerned with the nature of plasmid determined mercuric resistance. We thank in particular F. Cabello, S. N. Cohen, T. J. Foster, and J. L. Schottel in this regard.

The research was supported in part by Public Health

Service grants AI08062, GM14398, and RR7054 from the National Institutes of Health, and National Science Foundation grant PCM78-15787.

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