Effect of Catabolite Repression on the *mer* Operon

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The plasmid-determined *mer* operon, which provides resistance to inorganic mercury compounds, was subject to a 2.5-fold decrease in expression when glucose was administered at the same time as the inducer HgCl_2 . This glucose-mediated transient repression of the operon was overcome by the addition of cyclic AMP. Permanent catabolite repression of the operon was observed in the 1.6- to 1.9-fold decrease in expression in mutants lacking either adenyl cyclase (*cya*) or the catabolite activator protein (*crp*). The effect of the *cya* mutation on *mer* expression could be overcome by the addition of cyclic AMP at the time of induction. In addition to these effects on the whole cells of wild-type strains, we examined the effect of catabolite repression on the expression of the mercuric ion [Hg(II)] reductase enzyme, assayable in cell extracts, and on the Hg(II) uptake system, assayable in a mutant strain which lacked reductase activity. There was a two- to threefold effect of repression on the Hg(II) reductase enzyme assayable in vitro after induction under catabolite repressing conditions (either with glucose or in the *crp* and *cya* mutants). We did not find a similar repressing effect on the induction of the Hg(II) uptake system, which is also determined by the *mer* operon.

Genes determining resistance to mercury compounds are frequently found on plasmids in a variety of bacterial genera (26). The mechanism of resistance to inorganic mercury compounds commonly involves the inducible synthesis of an enzyme, the Hg(II) reductase (24, 27), which detoxifies the mercuric ion by reducing it to the volatile elemental form Hg(0) . In addition, an inducible mercuric ion uptake system has been observed to be a part of this system in plasmids of gram-negative bacteria (19, 25). Genetic studies of plasmids from both gram-positive and gram-negative bacteria (12, 19, 25, 30) define at least three functions of the mercury resistance (*mer*) operon: *merA*, the Hg(II) reductase; *merT*, the Hg(II) uptake function; and *merR*, the regulatory element. The evidence from studies with mutants of the prototypical IncFII plasmid, NR1 (R100), indicates that the system is under positive control (12).

The studies we report here demonstrate that the operon is also responsive to catabolite regulation mediated by the products of the *cya* (adenyl cyclase) and *crp* (catabolite activator protein [CAP]) genes. The magnitude of the effect which we see is similar to that previously reported for the ColE1 relaxation complex (16) and for plasmid-determined resistances to streptomycin (14) and chloramphenicol (11, 15), neither of which appears to be under any other specific type of regulation. The synthesis of the sex pilus of IncI plasmids is also affected by

cyclic AMP (cAMP) (13) and the CAP protein, and very recently, it has been reported that cAMP effects a very large stimulation (ca. 100-fold) in the synthesis of the plasmid-determined enterotoxin gene in *Escherichia coli* (17). Therefore, responsiveness to the effectors of the so-called global control systems appears to be common among plasmid-determined genes.

MATERIALS AND METHODS

Bacterial strains. All strains employed are listed in Table 1.

Transient glucose repression experiments. For experiments examining the short-term effects of glucose or cAMP or both on the induction of either the Hg(II) reductase activity or the Hg(II) uptake activity, the strain DU1040(pDU202), which is wild-type for the *mer* operon, was used. An overnight culture in tryptone broth (8 g of tryptone, Difco Laboratories, Detroit, Mich., and 5 g of NaCl per liter) was diluted 1:40 into the same medium and was grown with rapid shaking at 37°C to the midexponential phase (60 to 70 Klett units, Klett colorimeter, Wratten 54 filter). The culture was then split into several samples and induced either with HgCl_2 alone (at 1 μM for 15 min at 37°C followed by 1 to 5 μM for 15 min at 37°C) or with HgCl_2 and glucose (0.5%) or with HgCl_2 and cAMP (5 mM). The two-step induction procedure works best to minimize the toxic effects of HgCl_2 exposure and to maximize enzyme expression. The uninduced cultures were incubated along with the inducing ones until the induction protocol was completed (ca. 30 min; note that the average doubling time for all of the cultures described was between 40 and 50 min at 37°C). All

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid:	Genotype or phenotype	Reference or source
Bacterium		
DU1040	K-12 <i>lac his rpsL</i> Nal ^r	Foster et al. (12)
CA8000	K-12 Hfr Hayes <i>thi</i>	Brickman et al. (5)
CA8306	K-12 Hfr Hayes <i>cya thi</i>	Brickman et al. (5)
CA8445.1	K-12 Hfr Hayes <i>crp rpsL thi</i>	Brickman et al. (5)
J53	K-12 <i>pro met</i>	Bachmann (4)
J53 Nal ^r	K-12 <i>pro met</i> Nal ^r	This lab
Plasmid		
pDU202	Tra- <i>drd cml str sul fus mer</i> IncFII, derived from R100	Foster et al. (12)
pDU3315	From pDU202 by TnI insertion, <i>merA</i>	Foster et al. (12)

^a Nomenclature of the bacterial strains follows the recommendations of Demerec et al. (9), and nomenclature of the plasmids follows the recommendations of Novick et al. (20).

samples were then harvested by centrifugation and analyzed for whole-cell Hg(II) reductase activity, cell-free Hg(II) reductase activity, or Hg(II) uptake activity.

Cultivation of strains carrying *crp* and *cya* alleles. Since pseudorevertants of *crp* and *cya* strains arise at a high frequency (our unpublished data; W. Dobrogosz, personal communication), we found it necessary to freeze strains as soon as their correct construction had been verified and to use for each experiment only strains which were subcultured directly from the frozen stocks and which did not ferment lactose. For all experiments involving these mutants and their parent strain, cells were cultured in Luria broth (18) without glucose, rather than in tryptone broth, since these mutant strains grew very slowly or not at all in tryptone broth.

Enzyme assays. The assay of the Hg(II) reductase in whole cells and in vitro has been described previously (24, 25, 27), as has the assay of the Hg(II) uptake system (25). The isotope was quantitated by using the ¹⁴C setting in a Beckman LS7000 liquid scintillation spectrometer, and the data were reduced by using a BASIC algorithm on the Northstar Horizon micro-computer.

Reagents and isotopes. All chemicals were obtained from Fisher Scientific Co. and were of the highest purity available. Biochemicals and chromophoric reagents were obtained from Sigma Chemical Co., St. Louis, and were of the purest grade available. ²⁰³Hg was obtained from New England Nuclear Corp., Boston, Mass.

Assay of β -lactamase. β -Lactamase was assayed by using the chromogenic substrate Nitrocefin (21), which was a gift of Glaxo Research, Ltd., Middlesex, England.

Determination of flavin content. A washed-cell suspension (0.5 ml), prepared as for the Hg(II) reductase assay, was extracted with 2.5 ml of reagent-grade dimethyl sulfoxide. Cell debris was removed by centrifugation, and the flavin content of the supernatant was determined fluorometrically in a Turner Fluorometer (primary filter, 365 nm; secondary filter, 485 nm) by using flavin adenine dinucleotide (FAD) standards.

RESULTS

The first indication that the *mer* operon might be under catabolite repression came from our

observation that the level of constitutive enzyme expression varied as a function of the growth stage (see Fig. 3 of reference 25). We subsequently observed that there was a considerable variation in the specific activity of the enzyme inducible at different stages of growth. As cells approached the stationary phase in tryptone broth (a catabolite derepressing medium), both the constitutive and the inducible levels of the reductase increased markedly (25; Table 2). This increase was eliminated by including glucose in the growth medium (25). To avoid the nonspecific causes for this phenomenon which might arise in older cultures, all work described below was carried out with midexponential-phase cultures (unless otherwise indicated).

Unfortunately, it is not possible to do precise differential induction determinations in this system for several reasons. We currently do not have a gratuitous inducer of the structural genes which is effective in vivo; consequently, the inducer HgCl₂ is consumed as soon as the Hg(II) reductase synthesis begins, thus eliminating the

TABLE 2. Effect of glucose and of cAMP on induction of Hg(II) reductase in the wild-type strain^a

Age of culture at induction (Klett units)	Induction conditions		Sp act ^d
	Glucose ^b	cAMP ^c	
59	—	—	17.30
	+	—	8.29
	—	+	19.02
	+	+	15.84
120	—	—	38.96
	+	—	14.52
	—	+	78.37
	+	+	47.56

^a DU1040(pDU202).

^b Final concentration of glucose, 0.5%.

^c Final concentration of cAMP, 5 mM.

^d Nanomoles of Hg(II) reduced per minute per milligram of protein at 37°C.

possibility of examining induction kinetics with the inducer concentration held constant. Furthermore, because the inducer is toxic, it is not possible to work at very large excesses of the inducer, even with the wild-type resistant strains. Finally, even in the case of the supersensitive mutants, which are studied for their manifestation of the Hg(II) uptake function, one cannot be absolutely sure of the constancy of the HgCl₂ present in the medium, even though there is no Hg(II) reductase present in these strains. The reason for this is that mercuric ions, unlike sugars or amino acids, are readily adsorbed, in nonspecific ways, to cellular material. Therefore, as a culture of supersensitive cells grows in the presence of inducing, but subtoxic, levels of HgCl₂, the concomitant increase in cellular material will alter the concentration of the inducer. Because of these technical limitations, we used two different protocols to define the nature of the glucose effect on the *mer* operon. The protocols we used were: (i) short-term, "transient repression" experiments carried out with a host strain (DU1040), which is normal for the *crp* and *cya* alleles, and (ii) the examination of the specific activities of the various *mer* functions inducible in strains mutant for either adenyl cyclase (*cya*) or CAP (*crp*) (5). The experiments with the *crp* and *cya* mutants were carried out in a tryptone-yeast extract medium in which their rates of growth were similar to those of their parent strains. Concentrations of HgCl₂ were chosen so that there would be no detectable lag in the growth rate (as measured by Klett colorimeter) upon induction. We chose these conditions to allow reproducible comparisons which bear some physiological relation to previously characterized catabolite repressible systems (22, 23).

Transient glucose repression of Hg(II) reductase in intact cells. The addition of glucose and HgCl₂ resulted in a depression of the Hg(II) reductase activity of from 2- to 2.7-fold, depending on the stage of growth at which the cultures were induced (Table 2). cAMP added at the same time as the glucose and HgCl₂ overcame this depression of reductase induction both at the midexponential phase and at the early stationary phase. The addition of cAMP alone to the induced cells effected a marked stimulation of the HgCl₂-inducible Hg(II) reductase activity in the early stationary phase. These results suggest that the operon is responsive to "classical" catabolite repression (or, in this case, catabolite activation) mediated by adenyl cyclase and CAP (22, 23).

To examine this hypothesis more rigorously, we constructed derivatives of *crp* and *cya* strains and of their parent strain carrying plasmid pDU202 (an R100 derivative which has the

wild-type *mer* operon). The *crp* and *cya* strains which we used were deleted for their respective alleles and so should not have given true revertants (5). However, we found that both strains gave pseudorevertants at a rather high frequency; therefore, it was necessary to store newly constructed strains immediately and to work directly from these frozen stocks for each day's experiment. Our observations (Table 3) were that the inducible Hg(II) reductase specific activity was lowered by 1.6-fold in the *crp*⁻ strain and by 1.9-fold in the *cya*⁻ strain. In addition, cAMP markedly enhanced the induced level of the enzyme in the *cya*⁻ strain, but had no effect on the *crp*⁻ strains.

The current model of the *mer* operon holds that synthesis of the Hg(II) reductase and the Hg(II) uptake system are directed by mRNA, whose synthesis is initiated at a single promoter located in the *Eco*RI H fragment of plasmid NR1 (R100) (12, 26). If this is the case, and if the entire operon is under catabolite regulation, then we would expect that both activities would be affected to the same degree, either by transient glucose repression or by the *crp* and *cya* mutations. To test this hypothesis, we examined the cell-free Hg(II) reductase activity, determined by the wild-type *mer* operon carried by plasmid pDU202, and the Hg(II) uptake activity, determined by a *merA* derivative of this plasmid, pDU3315 (12). [Hg(II) uptake activity cannot be detected in a strain carrying the wild-type operon because the reductase reduces the Hg(II) to volatile Hg(0) as rapidly as it is taken up; no transient accumulation of Hg(II) can be observed (25).]

Transient catabolite repression of the Hg(II) reductase assayed in cell extracts. There was a decrease of approximately 2.24-fold in the Hg(II) reductase assayed in extracts of cells induced in glucose-supplemented tryptone broth (Table 4). cAMP, added with glucose, overcame this effect of glucose, and when cAMP was added without glucose, it markedly stimulated the induction of the enzyme. These data on transient catabolite repression of the cell-free

TABLE 3. Effect of cAMP on induction of Hg(II) reductase in intact cells of *cya* and *crp* mutants^a

Host	Sp act after induction ^b	
	-cAMP	+cAMP
CA8000 (<i>cya</i> ⁺ <i>crp</i> ⁺)	31.5	71.3
CA8306 (<i>cya</i> <i>crp</i> ⁺)	16.2	43.7
CA8445.1 (<i>cya</i> ⁺ <i>crp</i>)	20.0	20.3

^a Plasmid used, pDU202.

^b Nanomoles of Hg(II) reduced per minute per milligram of protein at 37°C.

TABLE 4. Comparison of Hg(II) reductase in intact cells and in cell extracts of the wild-type strain induced in the presence of glucose or cAMP^a

Induction conditions		Sp act ^b	
Glucose ^c	cAMP ^d	Intact cells	Cell extract
—	—	112.9	109.6
+	—	30.3	49.4
—	+	102.9	128.9
+	+	77.4	91.6

^a Wild-type strain, DU1040(pDU202).^b Nanomoles of Hg(II) reduced per minute per milligram of protein at 37°C.^c Final concentration, 0.5%.^d Final concentration, 5 mM.

activity were corroborated by those on the expression of the cell-free reductase activity by the *crp* and *cya* mutant strains (Table 5). The activity detectable in wild-type cells was 3.7-fold higher than that of the *cya* mutant and 3.2-fold higher than that of the *crp* mutant. Since these differences were similar to those seen in the intact cells, the effect in intact cells cannot be explained simply as an alteration in the uptake system alone leading to a masking effect on the intracellular reductase.

Effect of catabolite repression on Hg(II) uptake. The uptake of mercuric ions is measurable in intact cells of mutants which have lost the ability to synthesize an active Hg(II) reductase. These so-called supersensitive mutants still have a transport system which will take up Hg(II) from the medium and which is inducible by subtoxic concentrations of HgCl₂. Once induced, the supersensitive strains take up 2.5- to 5-fold more Hg²⁺ in a brief incubation than they do before induction (19, 25). Tables 6 and 7 show the relative inducibility of the Hg(II) uptake system of the mutant plasmid pDU3315, which has a supersensitive phenotype and a functional Hg(II) uptake system. Under conditions of transient catabolite repression, it did not appear that the addition of glucose had any negative effect (there may have been a positive effect) on the induction of the Hg(II) uptake system. There may have been a slight negative effect with the addition of cAMP, but the combination of cAMP and glucose gave results essentially equivalent to those for the addition of glucose alone. These results, which are very different from those seen with the reductase activity, are corroborated by the data in Table 7 on the effect of the *crp* and *cya* mutations on the induction of the Hg(II) uptake system. Here, again, we see that there was no effect on the induction ratio of the mutations affecting catabolite repression, although the overall uptake measurable in the *cya*⁻ strain was somewhat lower than that in the wild-type or *crp*⁻ host. Both types of data suggest that, unlike the case of the Hg(II) reduc-

tase induction, there is neither a repressing effect with the addition of glucose nor a stimulating effect of cAMP or CAP on the induction of the Hg(II) uptake system.

Is the effect on the Hg(II) reductase a result of decreased FAD levels? Since there was an effect of glucose and of cAMP or CAP on the Hg(II) reductase, but not on the Hg(II) uptake system, we considered the possibility that our results might arise from a decrease in cellular FAD levels. The Hg(II) reductase is an FAD-containing enzyme (24); Dills and Dobrogosz (10) have demonstrated that certain *crp* and *cya* mutations have only 10% of the wild-type FAD and 50% of the wild-type flavin mononucleotide (FMN). It was reasonable to think that the reductase apoenzyme might be synthesized without the effect of glucose, but that the active form of the enzyme could not form because of a lack of FAD. Since the effects we saw were most pronounced under conditions of transient repression, we examined flavin levels under those conditions. We found that the total extractable flavin content (measured fluorometrically) per milligram of cell protein was constant for all induction conditions, whereas the Hg(II) reductase activity was repressed by the addition of glucose and stimulated by cAMP (Table 2, data not shown). We also did not find significant differences between the flavin levels of the *crp* and *cya* mutants and their parent strains; the effect on flavin biosynthesis may be allele specific (W. Dobrogosz, personal communication).

Is the effect a result of copy number change in the plasmid? The copy number of R100 is stringently regulated to one to two copies per chromosome during exponential growth (31). For this reason, and because we saw an effect on one element of the operon [the Hg(II) reductase] and not on another [the Hg(II) uptake system] we reasoned that the effect was on the transcription of the Hg(II) reductase mRNA itself rather than on the gene copy number (i.e., on the plasmid copy number). To test this hypothesis, we examined the levels of β -lactamase in two strains carrying different derivatives of pDU202 into

TABLE 5. Comparison of Hg(II) reductase in intact cells and in cell extracts of *cya* and *crp* mutants^a

Host	Sp act ^b	
	Intact cells	Cell extract
CA8000 (<i>cya</i> ⁺ <i>crp</i> ⁺)	43.1	61.8
CA8306 (<i>cya</i> <i>crp</i> ⁺)	35.5	16.6
CA8445.1 (<i>cya</i> ⁺ <i>crp</i>)	19.5	19.4

^a Plasmid used, pDU202.^b Nanomoles of Hg(II) reduced per minute per milligram of protein at 37°C.

TABLE 6. Effect of glucose and cAMP on the induction of the Hg(II)-binding activity of DU1040(pDU3315)^a

Induction conditions		²⁰³ Hg bound ^b ± SD (pmol/mg of protein)		²⁰³ Hg bound induced/ ²⁰³ Hg bound uninduced
Glucose	cAMP	Uninduced	Induced	
—	—	309 ± 8	913 ± 83	2.95
+	—	316 ± 87	1,417 ± 2	4.48
—	+	180 ± 33	334 ± 18	1.85
+	+	353 ± 93	1,602 ± 113	4.50

^a All determinations done in duplicate.^b 15 min; 37°C.

which the ampicillin transposon Tn3 has been inserted outside of the *mer* operon. Since β -lactamase activity is directly related to the gene copy number of the *bla* locus, this enzyme activity is a useful diagnostic for the number of copies of the replicon carrying the *bla* locus.

We found that the specific activity of β -lactamase (measured spectrophotometrically) was constant and equivalent to the uninduced control for all conditions of induction. Hg(II) reductase levels examined in the same experiments demonstrated their usual responsiveness to glucose and cAMP (data not shown). We also measured the β -lactamase specific activity in the insertion mutant pDU3315 and found that under all the conditions used for induction of the Hg(II) uptake activity, the β -lactamase levels were constant and equivalent to the uninduced control (data not shown). We believe, therefore, that the effect we observed is on the transcription of the Hg(II) reductase itself. The implications of these observations for the current model of the *mer* operon are discussed below.

DISCUSSION

It is not unexpected that the mercury resistance system should be subject to catabolite repression. Recently, it has been shown that there are several chromosomally determined systems not immediately connected with carbohydrate metabolism which are subject to classical catabolite regulation (1–3, 6, 7, 10, 11, 28, 29). It has also been known for quite some time that two other plasmid-determined resistance systems, the chloramphenicol acetyl transferase

and the streptomycin adenylyl transferase, neither of which is inducible by its substrate, are both responsive to activation by cAMP and CAP (14, 15). Since the Hg(II) reductase consumes 1 mol of NADPH for every mole of mercuric ions it detoxifies, it can constitute a drain on the energy of the cell.

The inducibility of the Hg(II) reductase, measured both in whole cells and in cell extracts, is affected by transient repression (effected by glucose and relieved by cAMP) and also by permanent catabolite repression (resulting from the *crp* or *cya* mutations). Neither an alteration in the plasmid copy number nor a lack of flavin cofactor can account for the effects on reductase levels. The magnitude of the effects on the enzyme detectable in cell extracts is essentially equivalent to that in intact cells. In other words, the effects observable in whole cells probably result entirely from an alteration in the amount of active Hg(II) reductase synthesized. This hypothesis is substantiated by the observation that there is no marked effect of either transient or permanent catabolite repression (as defined above) on the inducibility of the Hg(II) uptake system.

The disparity in the effect of glucose on these two measurable parameters of the *mer* operon is problematical in terms of the current model of the operon, which holds that both the uptake system and the Hg(II) reductase arise from a single promoter. However, this model is based on limited data and there are currently no data to rule out the possibility that each of these two functions is synthesized from a different promoter.

TABLE 7. Effect of the *cya* and *crp* mutations on the induction of the Hg(II)-binding activity of plasmid pDU3315^a

Host	²⁰³ Hg bound ^b ± SD (pmol/mg of protein)		²⁰³ Hg ⁺ bound induced/ ²⁰³ Hg ⁺ bound uninduced
	Uninduced	Induced	
CA8000 (<i>cya</i> ⁺ <i>crp</i> ⁺)	454 ± 58	974 ± 57	2.14
CA8306 (<i>cya</i> <i>crp</i> ⁺)	231 ± 75	540 ± 24	2.34
CA8445.1 (<i>cya</i> ⁺ <i>crp</i>)	377 ± 113	903 ± 60	2.40

^a Results are the average of three separate experiments in which each binding assay was done in duplicate.^b 15 min; 37°C.

It is also possible that the apparent disparity between the effect of glucose or cAMP on Hg(II) uptake and on the reductase may simply be an artifact of the limited precision with which we can measure the uptake system compared with the reductase. Note that in supersensitive mutants (the only strains in which uptake can be measured), the activity of the Hg(II) uptake system is measured in ca. 500 pmol of Hg^{2+} bound in 15 min per mg of protein. The reductase activity measured in intact wild-type cells is, on the other hand, operating at a rate of ca. 70 nmol of Hg^{2+} reduced per min per mg of protein. In other words, what is measurable in the supersensitive mutants is less than 0.05% of the activity which must be functioning in the wild-type strain. The Hg(II) uptake system may, therefore, be one of facilitated diffusion. Results from Nakahara et al., who failed to demonstrate any definite energy source for the uptake system, fit this interpretation (19). The rate at which a facilitated diffusion system takes up mercuric ions will be dependent on the relative intracellular and extracellular concentrations of the ions. When the reductase is functioning to eliminate mercuric ions from the cell, the system perceives a large concentration difference between internal and external Hg(II) and is capable of functioning very rapidly. However, when the reductase is inactivated, as in the supersensitive mutants, the internal and external concentrations of Hg(II) quickly equilibrate. What is seen as uptake under these conditions may be only the saturation of nonspecific intracellular Hg(II)-binding sites such as sulfhydryl, carboxyl, or imino groups.

In support of this interpretation, we observed that 10 to 50 mM HgCl_2 (cold), 2-mercaptoethanol, EDTA, or sodium cyanide are all capable of eliminating ^{203}Hg uptake by supersensitive mutants when administered at the same time as the isotopic mercury, but only HgCl_2 can remove (chase) the ^{203}Hg when it is administered after binding has been allowed to take place. Nakahara et al. (19) also showed that the maximum binding of Hg^{2+} (after 40 min) by sensitive strains and by both induced and uninduced supersensitive strains is the same. However the initial rates of uptake, before saturation, are much higher for the induced supersensitive cells (19). These observations argue that the ultimate "sink" for ingested Hg(II) is the same in both sensitive and supersensitive cells and that the latter differ only in the initial rate at which they can saturate this sink, i.e., in the amount of diffusible carrier which they possess. Therefore, the Hg(II) uptake activity measurable in supersensitive mutants may not be a good indication of the levels of such activity which exist in intact, wild-type cells.

Unfortunately, because of this limitation in the measurement of the physiological activity of the Hg(II) uptake system, we cannot say definitively that the induction of the uptake system and of the reductase are affected differently by conditions of catabolite repression. So, although the disparity in the effects on the two functions casts some doubt on the validity of the current model of the operon, it is not a strong argument against it. A definitive answer to this question awaits, among other things, the characterization of the Hg(II) transport system.

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