

## Cloning and Expression in *Escherichia coli* of Chromosomal Mercury Resistance Genes from a *Bacillus* sp.

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**A 7.9-kilobase (kb) chromosomal fragment was cloned from a mercury-resistant *Bacillus* sp. In *Escherichia coli*, in the presence of a second plasmid carrying functional transport genes, resistance to  $\text{HgCl}_2$  and to phenylmercury acetate (PMA) was expressed. Shortening the cloned fragment to 3.8 kb abolished resistance to PMA but not to  $\text{HgCl}_2$ . In *Bacillus subtilis*, the 3.8-kb fragment produced mercuric reductase constitutively but did not produce resistance to  $\text{HgCl}_2$  or to PMA.**

Aerobic mercury-resistant bacteria detoxify mercury chiefly by two inducible enzymes, organomercurial lyase and mercuric reductase (17), acting, respectively, to cleave the carbon-mercury bond of certain organomercurial compounds and to reduce  $\text{Hg}^{2+}$  to volatile metallic mercury ( $\text{Hg}^0$ ) (18). Bacteria possessing both enzymes are said to exhibit broad-spectrum resistance (24); those possessing only mercuric reductase have narrow-spectrum resistance. DNA sequences coding for a metalloregulatory protein and for a mercury transport system are other vital components of the mercury resistance operon (*mer*) (15, 20). Mutants with a functional transport system but without a functional mercuric reductase gene, *merA*, are mercury hypersensitive (7).

In gram-positive *Staphylococcus aureus* (14, 24) and in gram-negative bacteria (6, 20), mercury resistance is extrachromosomally encoded. The *mer* operon subcloned from *S. aureus* plasmid p1258 resides on a 6.4-kilobase (kb) *Bgl*II fragment which expresses mercury resistance in *Bacillus subtilis* but not in *Escherichia coli* (10a). On the other hand, the resistance factors in the mercury-resistant ( $\text{Hg}^r$ ) *Bacillus* spp. which we previously isolated from heavy-metal-polluted aquatic sites are chromosomal (11). We now report the cloning of the mercuric reductase and organomercurial lyase genes from *Bacillus* sp. strain RC607, isolated from Boston Harbor. Expression of resistance to  $\text{HgCl}_2$  and to phenylmercury acetate (PMA) was obtained in *E. coli* transformed with a recombinant plasmid carrying the cloned RC607 DNA and a second compatible plasmid carrying a *mer* operon, derived from a gram-negative bacterium and functional for mercury transport but not for mercuric reductase activity.

The 4.0-kb *mer* operon of pDB7 (2) was excised with *Nco*I and *Stu*I and further digested with *Eco*RI (restriction enzymes and enzymes involved in nucleic acid metabolism were purchased from New England BioLabs, Inc., Beverly, Mass., and used as directed), and the resulting 2.8-kb fragment, with a truncated mercuric reductase gene but with functional mercury transport, was inserted into *Eco*RI-cut pSC101 (5). The recombinant plasmid, pYW22, was transformed (12) into competent cells of *E. coli* JM83 (27), and tetracycline-resistant transformants were selected (LB agar was used in all experiments involving the selection of colonies with specific resistances). The new strain, *E. coli* JM83(pYW22), was hypersensitive to  $\text{HgCl}_2$  ( $\text{Hg}^{\text{ss}}$ , see Table

1) and sensitive to 25  $\mu\text{M}$  PMA (Sigma Chemical Co., St. Louis, Mo.).

Chromosomal DNA was isolated (13) from *Bacillus* sp. strain RC607. DNA (1  $\mu\text{g}$ ) was digested with *Eco*RI and ligated to 0.5  $\mu\text{g}$  of *Eco*RI-cut pUC9 (23), a vector compatible with pSC101. JM83(pYW22) was transformed with the ligation mixture, and two transformants resistant to  $\text{HgCl}_2$  were obtained. Plasmid DNA extracted (3) from each of the two colonies contained pYW22 and a second recombinant plasmid carrying the cloned RC607 DNA.

The two recombinant plasmids which contained the cloned RC607 DNA were separated from pYW22. JM83 was transformed with plasmid DNA from the two mercury-resistant isolates, and plasmid DNA from ampicillin-resistant transformants was tested in 0.7% agarose gels for the absence of pYW22 and for the presence of a recombinant pUC9 vector. One such recombinant plasmid, pYW31, carried a 10.8-kb insert; the other plasmid, pYW32, on which subsequent studies focused, contained 7.9 kb of cloned DNA (Fig. 1).

Mercuric reductase activity in cell-extracts was assayed (8, 10) by monitoring the oxidation of NADPH (Sigma Chemical Co.) spectrophotometrically (340 nm). For enzyme induction,  $\text{HgCl}_2$  (2  $\mu\text{M}$  for *B. subtilis*; 5  $\mu\text{M}$  for *E. coli*; 25  $\mu\text{M}$  for RC607) was added 1 h before LB broth cultures were harvested at the end of the logarithmic growth phase. Assays were carried out for 4 min at 23°C in buffer (50 mM potassium phosphate [pH 7.0], 2 mM 2-mercaptoethanol) with 100  $\mu\text{M}$  NADPH in the presence or absence of 100  $\mu\text{M}$   $\text{HgCl}_2$ .

*E. coli* JM83 transformed with pYW32 was sensitive to both  $\text{HgCl}_2$  and PMA (Table 1). Although not mercury resistant, cells of JM83(pYW32) did produce substantial mercuric reductase activity constitutively (Table 1). Mercury and PMA resistance reappeared when pYW32 was again transformed into JM83(pYW22), indicating that pYW32 carried the structural genes for both mercuric reductase and organomercurial lyase and that the mercuric reductase of a gram-positive organism interacted with the gram-negative transport system. For subcloning experiments, large-scale plasmid DNA preparations were made (21). Digestion of pYW32 with *Eco*RI followed by ligase treatment resulted in a smaller plasmid, pYW40, which contained 6.2 kb of cloned DNA (Fig. 1). Plasmids pYW40 and pYW44 represent insertion of the 6.2-kb fragment in pUC9 in both orientations, as determined from double DNA digests with

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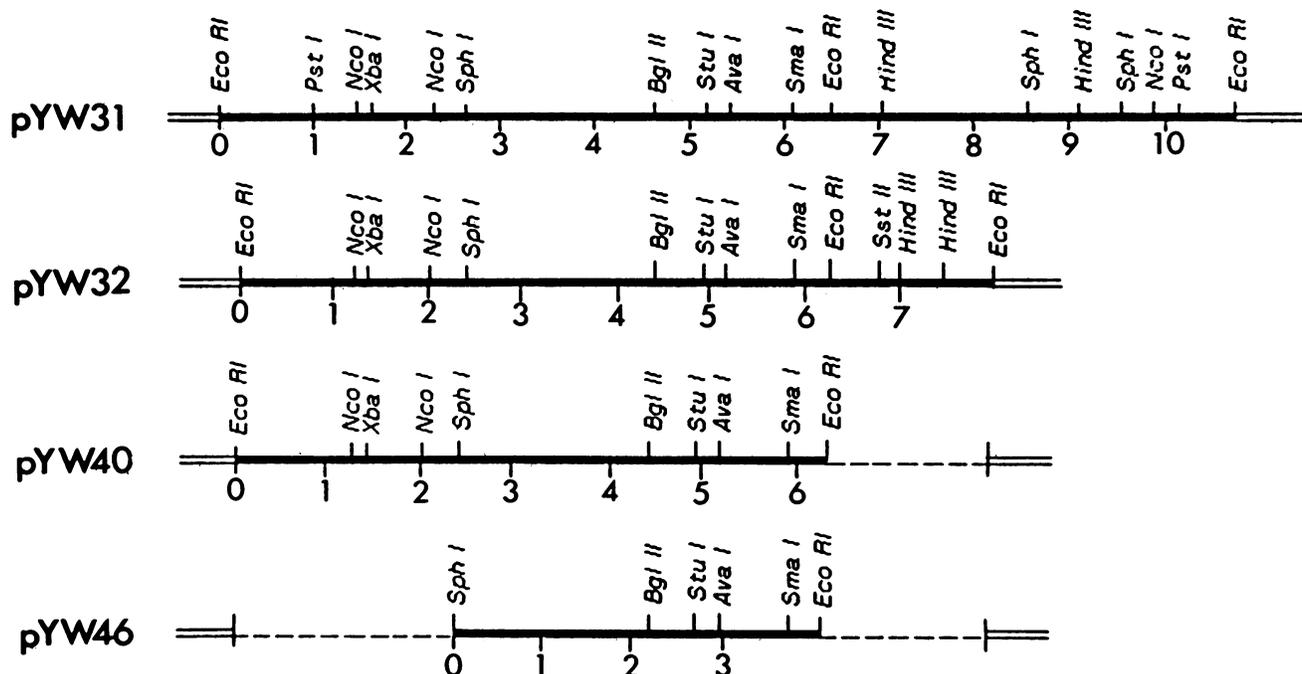


FIG. 1. Restriction maps of cloned and subcloned DNA sequences isolated from RC607. The top two lines represent the 10.8- and 7.9-kb inserts of pYW31 and pYW32. The smaller fragments were generated by digestion of pYW32 with *EcoRI*, followed by self-ligation, to yield pYW40. The 6.2-kb insert of pYW40 was further shortened by digestion of pYW40 with *EcoRI* and *SphI*, yielding pYW46.

*EcoRI* and *BglII* (data not shown). Although the enzyme activity of JM83(pYW44) was low (Table 1), cells carrying plasmids with the insert in either orientation produced mercuric reductase activity, suggesting the presence of a promoter.

Plasmid pYW40 was treated with *EcoRI* and *SphI*, followed by self-ligation. The resulting plasmid, pYW46, contained 3.8 kb of cloned DNA, produced mercuric reductase constitutively, and expressed mercury resistance in the presence of pYW22 (Table 1). However, cells carrying both pYW46 and pYW22 were not resistant to PMA. Thus, DNA deletion between the *EcoRI* and *SphI* sites (Fig. 1) resulted in a smaller plasmid lacking the PMA resistance sequence. Furthermore, digestion (Fig. 1) of plasmid pYW40 with *BglII* inactivated the mercuric reductase gene (*merA*), while digestion with *NcoI* inactivated the organomercurial lyase gene (*merB*). Thus, *merA* and *merB* have been localized in the cloned 6.2-kb DNA fragment of RC607 but are separated by at least 2 kb. In contrast, *merA* and *merB* of pI258 (14) are essentially contiguous (10a).

The constitutive expression of mercuric reductase activity by pYW32 and its subclones (Table 1) suggests that the regulatory region of the cloned DNA was either absent or not functional in *E. coli*. It can also be envisaged that the repressible mercuric reductase gene expressed in a multicopy plasmid titrates the repressor molecules.

To test the expression of the cloned DNA in *B. subtilis*, we linearized the DNA of pYW32 with *PstI* (at the pUC polylinker site) and ligated it to *PstI*-digested pPL608 (25), which then coded for  $Km^r$ . The chimeric plasmid (pYW60) DNA was first transformed into *E. coli* JM83(pYW22), to assure that mercuric reductase activity and mercury resistance were still expressed, and then transformed into competent cells (4) of *B. subtilis* 1A40 (*Bacillus* Genetic Stock Center, Ohio State University, Columbus). None of the  $Km^r$

transformants selected, e.g., 1A40(pYW60), was resistant to  $HgCl_2$  or PMA (Table 1), nor did any have detectable mercuric reductase activity. Attempts to induce mercuric reductase activity were unsuccessful, since even the highest

TABLE 1. Mercury resistance and mercuric reductase activity of *E. coli* and *Bacillus* strains

Bacterial strain	HgCl <sub>2</sub> resistance <sup>a</sup>		Mercuric reductase activity <sup>b</sup> (U/mg of protein)	
	Phenotype	MIC (μM)	Induced	Uninduced
<i>Bacillus</i> sp. strain RC607 <sup>c</sup>	Hg <sup>r</sup>	250	0.33	<0.01
<i>E. coli</i>				
JM83	Hg <sup>s</sup>	50	NT	<0.01
JM83(pDB7)	Hg <sup>r</sup>	150	0.08	<0.01
JM83(pYW22)	Hg <sup>ss</sup>	15	NT	<0.01
JM83(pYW22, pYW32) <sup>c</sup>	Hg <sup>r</sup>	125	0.43	0.41
JM83(pYW32)	Hg <sup>s</sup>	50	NT	0.46
JM83(pYW40)	Hg <sup>s</sup>	50	NT	0.45
JM83(pYW44)	Hg <sup>s</sup>	50	NT	0.07
JM83(pYW22, pYW46)	Hg <sup>r</sup>	125	0.46	0.46
<i>B. subtilis</i>				
1A40(pPL608)	Hg <sup>s</sup>	37.5	NT	<0.01
1A40(pYW60)	Hg <sup>s</sup>	37.5	NT	<0.01
1A40(pYW64)	Hg <sup>s</sup>	37.5	NT	0.42

<sup>a</sup> Growth inhibition required an MIC of >50 μM HgCl<sub>2</sub> for Hg<sup>r</sup>, ≤50 μM HgCl<sub>2</sub> for Hg<sup>s</sup>, and ≤15 μM HgCl<sub>2</sub> for Hg<sup>ss</sup>.

<sup>b</sup> One unit of mercuric reductase catalyzed Hg<sup>+</sup>- or Hg<sup>2+</sup>-dependent oxidation of 1.0 μmol of NADPH per min at 23°C. NT, Not tested.

<sup>c</sup> Resistant to 25 μM PMA.

nontoxic concentration of inducer was inadequate to induce mercuric reductase activity in the original isolate, RC607.

The transformation experiments were continued with the deletion plasmids pYW40 and pYW46 ligated to pPL608. Cells transformed with the chimeric plasmid containing pYW40 showed no mercuric reductase activity. Transformants carrying pYW46, e.g., 1A40(pYW64), had high levels of constitutively produced enzyme but were sensitive to HgCl<sub>2</sub> (Table 1). Since the *mer* operon of *S. aureus* was expressed in *B. subtilis* (10a), we had expected a DNA sequence cloned from a *Bacillus* sp. also to be expressed. Since none of the *B. subtilis* transformants showed resistance to HgCl<sub>2</sub>, the cloned fragment probably lacked a functional transport system. The fact that only the deleted insert of pYW46 produced constitutive mercuric reductase activity suggested that the 6.2- to 7.9-kb fragments contained a regulatory region upstream of the *SphI* site which, being nonfunctional in *E. coli*, functioned to inhibit mercuric reductase expression in *B. subtilis*.

For hybridization experiments, the 6.2-kb insert of pYW40 was excised with *EcoRI* and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (16). Using Southern blot transfers (19), we hybridized (1) the probe to *EcoRI*-cut chromosomal DNA from RC607, to plasmid DNA isolated from RC607, to plasmids pYW31, pYW32, and pDB7, and to the 6.4-kb *BglII* fragment containing the *S. aureus mer* operon cloned from pI258 (26). The probe hybridized to pYW31, pYW32, and a single fragment of *EcoRI*-cut RC607 DNA but showed no homology with RC607 plasmid DNA or with pDB7 (Fig. 2). A very faint band appeared with the *mer* operon of pI258 (a gift of R. A. Laddaga), suggesting a small region of homology (Fig. 2).

One of our aims in cloning the mercury resistance determinants from a gram-positive isolate was to assess the prevalence and distribution of such genes in the mercury-resistant, gram-positive population in polluted waters. Mercury-resistant colonies were isolated from metal-polluted aquatic environments by plating suitably diluted samples on agar containing 125  $\mu$ M HgCl<sub>2</sub>. The colonies were tested



FIG. 2. DNA-DNA hybridization. DNA samples were electrophoresed in a 0.7% agarose gel, transferred to a Zeta-Probe membrane (Bio-Rad Laboratories, Rockville, N.Y.), and probed with the <sup>32</sup>P-labeled 6.2-kb insert of pYW40. Lanes: A, chromosomal, *EcoRI*-cut RC607 DNA; B, plasmid DNA extracted from RC607; C, *EcoRI*-cut pYW31; D, *EcoRI*-cut pYW32; E, *PstI*-cut pDB7 *mer* operon from *E. coli*; F, 6.4-kb *BglII* fragment of pI258; G, phage lambda DNA digested with *HindIII*.

with the <sup>32</sup>P-labeled probe by colony hybridization (9). Of 2,000 colonies examined, 92 (4.6%) reacted with the probe. When the water samples were heated (80°C, 10 min) prior to plating, 216 CFU (11%) survived. Of the survivors, presumably sporeformers, 54 colonies (25%) reacted with the probe (data not shown). Thus, the chromosomal resistance determinants of RC607 can be detected among diverse mercury-resistant, gram-positive natural isolates. However, the <sup>32</sup>P-labeled probe showed no homology with numerous other mercury-resistant *Bacillus* spp., including those described by Izaki (10) and Timoney et al. (22).

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