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# Expression of Bacterial Mercuric Ion Reductase in *Saccharomyces cerevisiae*

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**The gene *merA* coding for bacterial mercuric ion reductase was cloned under the control of the yeast promoter for alcohol dehydrogenase I in the yeast-*Escherichia coli* shuttle plasmid pADH040-2 and transformed into *Saccharomyces cerevisiae* AH22. The resulting transformant harbored stable copies of the *merA*-containing hybrid plasmid, displayed a fivefold increase in the MIC of mercuric chloride, and synthesized mercuric ion reductase activity.**

Mercuric ion resistance is the most extensively studied heavy metal resistance and has been found in a wide range of gram-positive and gram-negative prokaryotes. The mechanism of mercuric ion resistance is the reduction of $Hg^{2+}$ to $Hg^0$ by the intracellular enzyme mercuric ion reductase (MerA) and then the volatilization of the relatively nontoxic metallic mercury. However, *mer* determinants may encode up to six gene products in addition to MerA in gram-negative bacteria: the activator/repressor protein MerR, the specific $Hg^{2+}$ carrier MerT, the periplasmic $Hg^{2+}$-binding protein MerP, the second $Hg^{2+}$ transport system MerC, the organomercurial lyase MerB, and the MerD protein, which is involved in the regulation of *mer* (9, 12, 15, 17, 22). The *mer* determinant cloned from *Thiobacillus ferrooxidans*, however, encodes only two proteins, MerA and MerC, which confer mercuric ion resistance to *Escherichia coli* (21). Therefore, along with MerA, at least one additional component appears to be involved in full expression of mercuric ion resistance in prokaryotes.

After the development of a procedure for the transformation of yeasts (10), the bacterial antibiotic resistance gene *cat* was functionally expressed in *Saccharomyces cerevisiae* (7), opening the possibility of investigating the action of bacterial genes in a eukaryotic cellular environment. In this study, we describe the functional expression of mercuric ion reductase in *S. cerevisiae*.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *E. coli* NM522 and S17-1 were used as plasmid hosts and grown in Luria broth (LB) (20) at 37°C. *S. cerevisiae* strains were cultivated at 30°C in rich medium (yeast extract-peptone-dextrose [YPD]) or minimal medium (1) containing 2% (wt/vol) glucose and 10 μg of histidine per ml. Complex media (LB, YPD) were solidified with 1.5% (wt/vol) agar, and minimal media were solidified with 2% (wt/vol) agar. Turbidity measurements were done with a Klett-Summerson photoelectric colorimeter (no. 54 Kodak Wratten filter; Eastman Kodak Co., Rochester, N.Y.).

**Metal resistance.** To estimate the MIC range, defined as the lowest concentration of metal salts at which no colony formation occurs, cells were streaked on minimal agar containing 5 to 50 μM $HgCl_2$ or on YPD agar containing 100 to 1,000 μM $HgCl_2$ and incubated for 3 days at 30°C. For the determination of the exact MICs, yeast cells were pregrown for 48 h in liquid minimal medium, diluted appropriately, and plated onto minimal agar containing 1, 2, 3, 5, 7, 10, 20, 30, or 50 μM $HgCl_2$. The colonies were counted after 3 days of growth at 30°C.

**Genetic techniques.** Standard molecular genetic techniques were applied (1, 20). For the preparation of total yeast genomic DNA (6), cells were grown overnight in YPD. Of this culture, 2 ml was harvested by centrifugation and suspended in 0.5 ml of KCl solution (0.4 M), and 25 μl of Zymolase 60000 was added. After 30 min of incubation at 30°C, 100 μl of EDTA (0.5 M) and 12 μl of Triton X-100 were added and cells were lysed for 20 min at 70°C. After centrifugation, the DNA-containing supernatant was purified with QIAGEN tips (Quiagen Inc., Chatsworth, Calif.) by following the supplier's instruction. Transfer of total genomic or plasmid DNA to Biodyne B nylon membranes (pore size, 0.45 μm; Pall Filtrationstechnik, Dreieich, Germany) and hybridization were performed as described elsewhere (20, 25). The 2.3-kb *merAD*-containing *Hin*dIII fragment of pECD384 used as a DNA probe was isolated from an 0.8% (wt/vol) agarose gel by using QIAEX (Quiagen) and biotinylated with a nick translation labelling kit (BRL BluGene; GIBCO-BRL, Bethesda, Md.).

**Transformation of *S. cerevisiae*.** Plasmid DNA (10 μg of DNA per transformation) was introduced into yeast cells by using lithium acetate (1). No additional carrier DNA was included in the transformation mixtures, which were plated onto complete leucine dropout medium (1), a minimal salts medium containing glucose and all amino acids except leucine. However, amino acids were supplied at a 10-fold higher concentration than described in the protocol (1). Plasmids were recovered from yeast cells by the glass bead procedure (1) and used to retransform *E. coli* S17-1.

**Mercuric ion reductase assay.** The activity of the mercuric ion reductase (reduced NADP:mercuric ion oxidoreductase) was determined spectrophotometrically (19) at 365 nm. Yeast cells were grown in YPD medium at 30°C, and *E. coli* cells were grown in LB medium at 37°C; both were grown overnight with shaking. Cells were harvested by centrifugation and resuspended in buffer composed of 50 mM sodium phosphate (pH 7.5), 0.5 mM EDTA, 0.2 mM magnesium

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TABLE 1. Strains and plasmids

| Strain or plasmid | Relevant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* NM522 | | Pharmacia |
| *E. coli* S17-1 | | 24 |
| *S. cerevisiae* AH22 | *LEU2-3 LEU2-112 HIS4-519 CAN1 cir*$^+$ | 10 |
| *S. cerevisiae* AH22-1 | Leu$^-$ His$^-$, "cured" derivative of AH22(pECD384) | This study |
| Plasmids | | |
| pDU1003 | *mer tet* | 16 |
| pTZ18R | *bla* | Pharmacia |
| pADH040-2 | *E. coli-S. cerevisiae* shuttle vector, *adh* promoter, *leu2 bla* | 18 |
| pBR322 | *bla tet* | 4 |
| pBR325 | *bla tet cat* | 3 |
| pBluescript SK$^+$ | *bla* | Stratagene |
| pECD377 | Vector plasmid, *cat*, pUC19 cloning sites | This study |
| pECD379 | *merAD* with a *Sna*BI site upstream cloned into pECD377 | This study |
| pECD380 | *bla* gene from pBR322 cloned into pECD379 | This study |
| pECD381 | ADH promoter cloned into pBluescript | This study |
| pECD382 | *Cla*I fusion of pECD381 and pECD379 | This study |
| pECD383 | Partial fusion of pECD382 and pECD380 | This study |
| pECD384 | *merAD* cloned into pADH040-2 | This study |

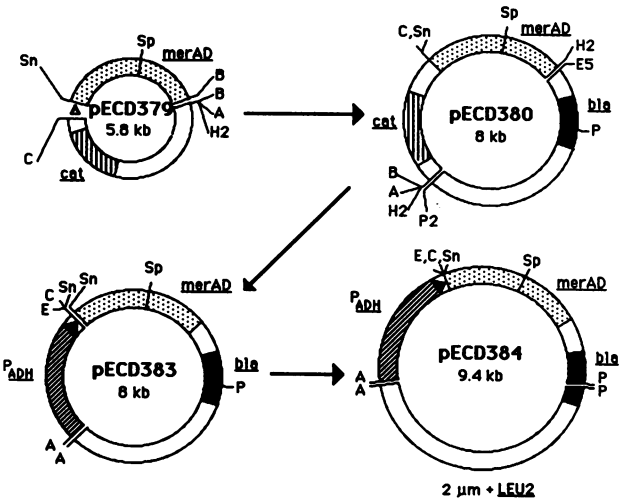


FIG. 1. Construction of plasmid pECD384. Maps of the four plasmids pECD379, pECD380, pECD383, and pECD384 are shown. The arrows indicate the path of construction. Regions of the genes encoding mercuric ion reductase and MerD (*merAD*), chloramphenicol acetyltransferase (*cat*), beta-lactamase (*bla*), and the promoter for the yeast alcohol dehydrogenase ($P_{ADH}$; an arrowhead indicates the direction of transcription) are given. The triangle in plasmid pECD379 marks the position of a small *Hin*dIII-*Pvu*I fragment which was deleted from pECD378, leading to pECD379 after mung bean nuclease treatment and religation. This step created an *Sna*BI site as indicated; the *Cla*I site originally adjacent to the *Hin*dIII site was left intact and shares 1 bp with the *Sna*BI site. Only the restriction sites relevant for construction are indicated: B, *Bam*HI; Sp, *Sph*I; C, *Cla*I; H2, *Hin*dII; A, *Xba*I; Sn, *Sna*BI; P2, *Pvu*II; E5, *Eco*RV; P, *Pst*I.

acetate, and 0.1% (vol/vol) 2-mercaptoethanol. Cells were subjected to ultrasonic disruption (Branson, Danbury, Conn.). To decrease the interfering activity of particulate NADPH oxidase, the membrane fraction was separated from the soluble extract by centrifugation for 60 min at 140,000 × *g*. The reaction mixture (total volume of 1 ml) contained 50 mM sodium phosphate (pH 7.5), 0.5 mM EDTA, 0.2 mM magnesium acetate, 0.1% (vol/vol) 2-mercaptoethanol, 200 μM NADPH (Boehringer, Mannheim, Germany), 240 μM $HgCl_2$, and approximately 220 μg of protein from the soluble fraction of the extract. The high mercuric ion concentration (240 μM) in the reaction mixture gave the best results with yeast cells. $Hg^{2+}$-dependent NADPH oxidation was measured at 37°C. One unit (U) is defined as the enzyme activity able to oxidize 1 μmol of NADPH per min. Protein was determined by the Bradford method (5).

## RESULTS

**Construction of plasmid pECD384.** The *merAD* region was cloned from plasmid pDU1003 (16) as a 2,255-bp *Nae*I-*Hin*dII fragment into pTZ18R. Analysis of the *merA* sequence (2, 13, 14) revealed an out-of-frame ATG codon immediately upstream from the translational start of *merA* (CGATGGTATG). To avoid problems in eukaryotic translation, this first ATG was changed to CGATCGTATG by oligonucleotide-directed mutagenesis (11), leading to the generation of a *Pvu*I recognition site. Further cloning steps were performed with a 4.3-kb vector plasmid (pECD377) encoding chloramphenicol resistance (Cm$^r$). Plasmid pECD377 had been constructed by ligating a *Hin*dII-*Sph*I fragment from plasmid pBR325 with the *cat* gene and a *Sph*I-*Fsp*I fragment of plasmid pTZ18R including a part of the pUC19 polylinker and the ColE1 origin of replication. Subsequently, the altered *merAD* fragment with the newly introduced *Pvu*I site was cloned into plasmid pECD377 with *Bam*HI. Digestion with *Pvu*I and *Hin*dIII and treatment with mung bean nuclease deleted the upstream region (indicated by Δ in Fig. 1) of the *merA* gene and generated a *Sna*BI site in the resulting plasmid pECD379 (Fig. 1).

The *bla* gene conferring ampicillin resistance was cloned from plasmid pBR322 as an *Eco*RV-*Pvu*II fragment into the *Hin*dII site of plasmid pECD379, yielding plasmid pECD380 (Fig. 1). The *Xba*I-*Sna*BI fragment of pECD380 containing the *cat* gene was replaced by the yeast promoter for the alcohol dehydrogenase I gene. The yeast promoter, which originated from plasmid pADH040-2 (18), was cloned as an *Eco*RI-*Xba*I fragment into pBluescript SK$^+$, excised as a *Cla*I-*Xba*I fragment, inserted into pECD379, and finally cloned as an *Sna*BI-*Xba*I fragment into pECD380, leading to plasmid pECD383 (Fig. 1).

In the last step of the construction, plasmid pECD384 was generated by ligating the *Xba*I-*Pst*I fragment of pADH040-2 (18) containing the 2μm plasmid origin of replication, the *Leu2* gene (8), and the downstream part of the *bla* gene with the *Xba*I-*Pst*I fragment of pECD383 containing the yeast promoter, the *merA* gene, and the upstream part of the *bla* gene (Fig. 1).

**Replication of pECD384 in *S. cerevisiae* AH22.** Plasmids pECD384 and pADH040-2 were isolated and used to transform the leucine-auxotrophic *S. cerevisiae* strain AH22, yielding about 200 transformants per μg of DNA. Plasmid DNA was isolated from six individual yeast transformants and used to retransform *E. coli* S17-1. The *Eco*RI restriction

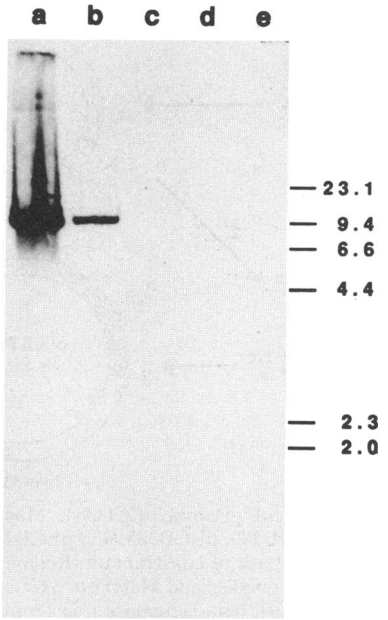


FIG. 2. Presence of the *merA*-containing hybrid plasmid in *S. cerevisiae*. Total genomic DNA from the yeast strains AH22 (pECD384) (lane b), AH22-1 (plasmid cured; lane c), AH22 (pADH040-2) (lane d), and AH22 (lane e) and plasmid DNA from *E. coli* S17-1(pECD384) (lane a) were isolated, digested with *Xba*I, separated on an 0.8% agarose gel, and blotted onto a nylon membrane. The digests were hybridized with the biotinylated 2.3-kb *Hin*dIII fragment of pECD384 containing the *merA* determinant. The sizes (in kilobases) of *Hin*dIII-digested λ DNA fragments are marked on the right.

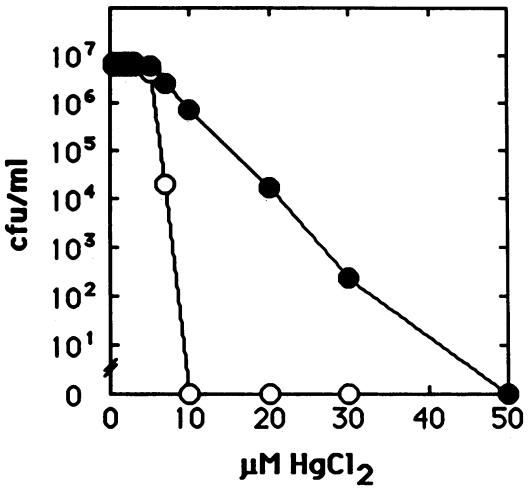


FIG. 3. Resistance to mercuric ion of MerA-containing *S. cerevisiae* strains. Strains AH22(pECD384) (●) and AH22(pADH040-2) (○) were grown for 48 h at 30°C in minimal medium, diluted, and plated onto minimal medium containing various concentrations of $Hg^{2+}$. After 3 days of growth at 30°C, the number of CFU was determined and plotted against the respective mercuric ion concentration.

pattern of plasmid DNA isolated from ampicillin-resistant *E. coli* transformants was identical to the pattern of the original plasmid pECD384 (data not shown).

One of the *S. cerevisiae* transformants, AH22(pECD384), was selected for further studies and kept on mineral salts medium containing glucose and histidine. Under these conditions, plasmid pECD384 was stably maintained. However, under nonselective conditions the plasmid was lost. This was indicated by the occurrence of leucine auxotrophs after cells of AH22(pECD384) had been grown to stationary phase for five cycles in YPD medium. One of the auxotrophs was named AH22-1.

Total DNA from *S. cerevisiae* AH22(pECD384), AH22-1, AH22(pADH040-2), and AH22 and plasmid DNA from *E. coli* S17-1(pECD384) were isolated, digested with *Xba*I, and used in Southern blot DNA-DNA hybridization, with the 2.3-kb *merAD* fragment used as a probe (Fig. 2). Strong signals corresponding to a 9.4-kb *Xba*I fragment (lanes a and b) and weaker signals corresponding to 8- and 7-kb fragments (lane d) were identified. The faint bands are apparently the result of a nonspecific reaction.

**Phenotypic resistance to mercuric ion in *S. cerevisiae*.** All transformants of *S. cerevisiae* AH22 carrying plasmid pECD384 were resistant to $Hg^{2+}$, with MICs of 30 μM $HgCl_2$ on minimal agar and 1 mM $HgCl_2$ on YPD agar. In contrast, the recipient strain, the vector-containing control strain, and pECD384-cured derivatives were sensitive to $Hg^{2+}$, exhibiting MICs of 6 ± 1 μM $HgCl_2$ on minimal agar and 600 μM $HgCl_2$ on YPD agar.

When the plating efficiencies of AH22(pECD384) and AH22(pADH040-2) on $Hg^{2+}$-containing mineral salts medium were compared (Fig. 3), the *merA*-free strain did not form colonies at $Hg^{2+}$ concentrations above 10 μM, whereas the *merA*-harboring transformant maintained its colony-forming ability up to 30 μM $Hg^{2+}$, although at a reduced efficiency.

In liquid medium, strain AH22(pECD384) grew after a lag phase even at 10 μM $HgCl_2$. In contrast, strain AH22 (pADH040-2) ceased to grow at 5 μM $HgCl_2$ and growth was strongly inhibited at 3 μM $HgCl_2$ (data not shown).

**Activity of mercuric ion reductase in *S. cerevisiae*.** Extracts prepared from AH22(pECD384) showed clear stimulation of NADPH oxidation upon the addition of $Hg^{2+}$, while extracts prepared from AH22(pADH040-2) displayed an $Hg^{2+}$-dependent inhibition of NADPH oxidation (Table 2). From these results, it is concluded that mercuric ion reductase is functionally expressed from plasmid pECD384 in strain AH22. The activity of mercuric ion reductase in the mem-

TABLE 2. Mercuric ion reductase activity in *S. cerevisiae*

| Strain | Mercuric ion reductase activity[a] | |
|---|---|---|
| | With $HgCl_2$ | Without $HgCl_2$ |
| *S. cerevisiae* AH22(pECD384)[b] | 11.5 ± 2.5 (3) | 3.2 ± 0.4 (3) |
| *S. cerevisiae* AH22(pADH040-2)[b] | 2.8 ± 0.2 (2) | 3.8 ± 0.2 (2) |
| *E. coli* S17-1 (pDU1003)[c] | 188 ± 11 (2) | 3.4 ± 0.3 (2) |
| *E. coli* S17-1 (pDU1003)[d] | 2.6 ± 0.2 (2) | 3.3 ± 0.6 (2) |

[a] NADPH-oxidizing activity of crude extracts prepared from both yeast strains in the presence of 200 μM NADPH and in the presence or absence of 240 μM $Hg^{2+}$ is given in micromoles of NADPH oxidized per minute per gram of protein. Values are means based on different numbers of determinations (given in parentheses).

[b] Cultivated overnight in YPD medium without $Hg^{2+}$.

[c] An overnight culture in LB containing tetracycline was diluted 25-fold with LB and incubated with shaking for 2.5 h at 37°C. $HgCl_2$ was added to a final concentration of 1 μM, and incubation was continued for 15 min.

[d] Cultivated overnight in LB without $Hg^{2+}$.

brane-free supernatant of AH22(pECD384) was 8 U/g of protein; this value was calculated from the difference between the rates of NADPH oxidation before and after the addition of $Hg^{2+}$.

The MerA activity of *E. coli* S17-1(pDU1003) was 185 U/g of protein in cells induced for 15 min with 1 μM $Hg^{2+}$, while NADPH oxidation was inhibited in uninduced cells of *E. coli* S17-1(pDU1003). Thus, the activity of mercuric ion reductase in yeast cells was 4.3% of the activity obtained with induced cells of *E. coli* containing the complete *mer* operon (Table 2).

## DISCUSSION

This paper describes the first example of a eukaryotic organism transformed to a phenotypic heavy-metal resistance by functional expression of a bacterial metal resistance gene. The *merA* gene encoding mercuric ion reductase was cloned under the control of a yeast promoter on plasmid pECD384, which is able to replicate in *S. cerevisiae*. The presence of the *merA*-containing plasmid in *S. cerevisiae* was demonstrated by retransformation into *E. coli* and Southern DNA-DNA hybridization. Plasmid pECD384 was stably maintained in *S. cerevisiae*: the occurrence of plasmid-free descendants took at least three cycles of growth under nonselective conditions.

All yeast strains containing pECD384 were about five times more resistant to $Hg^{2+}$ on minimal agar than was the parent strain. Moreover, pECD384-containing yeast strains exhibited mercuric ion reductase activity in the crude extract. Thus, *S. cerevisiae* was transformed to mercury resistance by the expression of the prokaryotic *merA* gene.

The *merA* fragment cloned in pECD384 additionally contained the *merD* gene, which is located downstream of *merA*. However, the *merD* gene should not be translated in *S. cerevisiae* from a polycistronic *merAD* mRNA, and, moreover, its expression should have no effect on yeast cells, since MerD is proposed to be involved in the regulation of the *mer* operon in *E. coli* (15, 17, 23).

The *merA* gene was previously functionally expressed in mammalian cell lines, as determined by MerA activity (26). However, no phenotypic resistance was acquired. For the expression of the *merA* gene in this eukaryotic system, the presence of a terminator was essential (26).

The specific activity of mercuric ion reductase in crude extract of *S. cerevisiae* was 8 μmol of NADPH per min per g of protein and is much lower than the value of 185 μmol of NADPH per min per g of protein obtained with *E. coli*. Nevertheless, this enzyme activity resulted in a fivefold increase in resistance to $Hg^{2+}$ when cells were grown on minimal medium. Mercuric ion tolerance of both the sensitive and the resistant yeast cells is much higher on complex medium because of the presence of metal-chelating components, e.g., amino acids, and is much higher than that of sensitive *E. coli* cells (21).

It is remarkable that expression of the MerA protein alone results in mercury resistance in *S. cerevisiae*; mercury resistance in bacteria usually requires the expression of several genes (22). Expression of the *Thiobacillus merA* gene in *E. coli* resulted in a twofold increase in mercury resistance, which was enhanced to fivefold by the additional expression of the *merC* gene (21). Therefore, enhancement of a specific $Hg^{2+}$ transport may result in enhancement of resistance to mercuric ion in yeast cells containing the *merA* gene.

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