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Role of Cell Wall in *Saccharomyces cerevisiae* Mutants Resistant to Hg^{2+}

BUN-ICHIRO ONO,* HIDEKI OHUE, AND FUMIYO ISHIHARA

Laboratory of Environmental Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700, Japan

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Hg^{2+}-resistant mutants were isolated from *Saccharomyces cerevisiae*. Although they were very much like the parental strains in terms of colony-forming ability, they grew faster than the parental strains in the presence of sublethal doses of Hg^{2+}. The Hg^{2+}-resistant mutations were dominant. They were centromere linked and were divided into two groups by means of recombination; one of the mutations, designated *HGR1-1*, was mapped on chromosome IV because of its linkage to the *TRP1* locus. The Hg^{2+}-resistant mutants took up Hg^{2+} as much as, or slightly more than, the parental strains did. The mutants and parental strains retained only about 5 and 15%, respectively, of the cell-associated Hg^{2+} after removal of the cell wall; therefore, the mutants had less spheroplast-associated Hg^{2+} than did the parental strains. These results indicate that the cell wall plays an important role in protection against Hg^{2+} by acting as an adsorption filter and that the mutations described confer Hg^{2+} resistance by increasing the Hg^{2+}-binding capacity of the cell wall.

For a better understanding of the toxicity of mercury and mechanisms of biological defense against mercury, studies at the cellular and subcellular levels are indispensable. *Saccharomyces cerevisiae* is suitable for such studies because it is easily manipulated genetically and readily analyzed biochemically. Singh and Sherman (17, 18) isolated mutants resistant to methylmercury and showed that the methylmercury-resistant mutations arose in only the *MET2* and *MET15* loci. On the other hand, mutants resistant to inorganic mercury were obtained by training; i.e., a wild-type strain was cultured in medium containing a sublethal concentration of mercury, and then the mercury concentration was gradually increased as the cells grew (5, 22). However, the resultant mutants were so unstable that they quickly lost resistance when they were cultured without mercury.

We have been studying the toxicity of Hg^{2+} and the biological defense against Hg^{2+} by using *S. cerevisiae*. It has been shown that the Hg^{2+} sensitivity of a certain strain is caused by the joint action of two mutations (10). One of them blocks tyrosine biosynthesis, while the other blocks tyrosine uptake by enhancing catabolite repression of the tyrosine uptake system (11, 13). Hg^{2+} promotes depletion of cellular tyrosine by inhibiting the tyrosine uptake system (13). To search for other biological effects of Hg^{2+}, we attempted to obtain stable Hg^{2+}-resistant mutants. In this report, we describe the isolation and partial characterization of the mutants. From these results, we discuss a role of the cell wall in protection against Hg^{2+} in *S. cerevisiae*.

MATERIALS AND METHODS

Growth media, strains, and isolation of mutants. YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose (15). For solid media, 2% agar was added. Mutants were isolated from strain EH1-4A (*MATα leu2-1 met8-1 gal2 msm1-2*) and EH1-4B (*MAT***a** *leu2-1 met8-1 his5-2 MSM1-1*). Cells were grown to early stationary phase, harvested, and spread on YPD medium at a density of about 5×10^6 per plate. The plates were irradiated by UV light at a dose of approximately 50 J/m^2. A filter paper disk (5 mm in diameter) was placed at the center of each plate, and 2.1 mg of $HgCl_2$ in solution was applied to each disk. The plates were incubated at 30°C.

Tests for Hg^{2+} resistance. In the filter paper disk method, cells were suspended in water at a density of about 10^4 per ml, and the cell suspension was streaked radially on agar YPD medium. Hg^{2+} was applied as described above. Growth was scored after appropriate incubation periods. For growth kinetics, cells were inoculated in liquid YPD medium with or without 220 μM $HgCl_2$, and the culture was incubated with rotary shaking at 60 rpm. Growth was monitored with a Klett-Summerson spectrophotometer equipped with a red filter. Colony-forming ability was tested by spreading cells on YPD medium containing various concentrations of Hg^{2+}.

Genetic procedures. Standard yeast genetic procedures were used (15). Genetic markers were scored as described previously (9). Linkage was evaluated by statistical significance of deviation from a 1:1 ratio of parental-ditype to nonparental-ditype tetrads. Genetic distances were expressed in centimorgans (12).

Preparation of spheroplasts. Cells, radioactively labeled (described below) or not, were washed and suspended in 0.1 M sodium phosphate buffer, pH 5.7, containing 26 mM mercaptoethanol and 60 mM EDTA. The cell suspension was incubated at 30°C for 10 min. The cells were collected, suspended in 0.1 M phosphate buffer (pH 5.7) containing 26 mM mercaptoethanol, 0.6 M KCl, and 20 μg of Zymolyase 60000 per ml, and incubated at 30°C for 30 min. The spheroplasts were collected, washed three times with 0.1 M phosphate buffer (pH 5.7) containing 0.6 M KCl, and suspended in the same buffer. The density of spheroplasts was measured under a microscope, using a hemacytometer.

Uptake of Hg^{2+} by cells or spheroplasts. Cells in mid-logarithmic phase were suspended in 0.1 M sodium phosphate buffer (pH 5.7) at a density of 2×10^6 per ml (cell density was measured by using a hemacytometer). $^{203}HgCl_2$ (350 Ci/mol) was added to the cell suspension, to a final concentration of 17.4 nM; at this concentration, Hg^{2+} was not lethal to cells at all. The reaction mixture was incubated at 30°C, and 0.5-ml portions were removed at various

* Corresponding author.

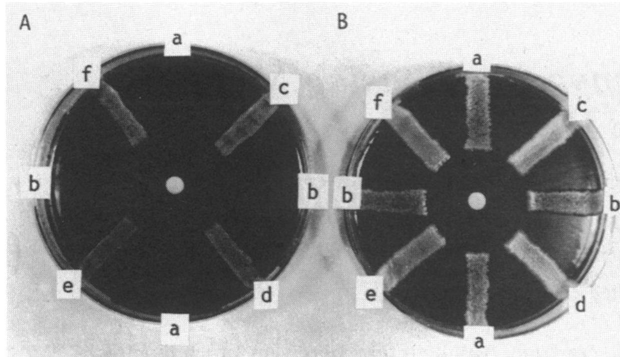

FIG. 1. Filter paper disk test. Parental strains EH1-4A (a) and EH1-4B (b) and mutants derived from them, R4A-1 (c), R4A-2 (d), R4B-1 (e), and R4B-2 (f), were inoculated on YPD plates. A filter paper disk was placed at the center of each plate, and $HgCl_2$ (2.1 mg) in solution was applied onto the disk. The plates were incubated at 30°C for 4 (A) and 7 (B) days.

intervals. Cells were collected and washed three times with 1 ml of the incubation buffer. Radioactivity was then measured with a γ-dosimeter (model JDC-751; Aloka, Tokyo, Japan). Spheroplasts were treated in the same way except that 0.6 M KCl was added to the incubation and washing buffers.

Chemicals. Yeast extract and peptone were products of Difco Laboratories (Detroit, Mich.). Amino acids were products of Nakarai Chemicals (Kyoto, Japan). Zymolyase 60000 was purchased from Seikagaku Kogyo, Ltd. (Tokyo, Japan). $HgCl_2$ and $^{203}HgCl_2$ were purchased from Katayama Chemicals (Osaka, Japan) and Daiichi Kagakuyakuhin Co., Ltd. (Tokyo, Japan), respectively. Other chemicals used were of analytical grade.

RESULTS

Isolation of mutants. After 5 days of incubation of the selective plates (see Materials and Methods), a clear growth inhibition zone was formed at the center of each plate. Outside the growth inhibition zone, colonies grew. Although most of the colonies were much smaller than usual, a few were relatively large. These larger colonies were picked and subcloned to obtain pure clones. In this way we obtained 18 mutants, including 2 spontaneous mutants. The mutants were tested by the filter paper disk method. After 4 days of incubation, the mutants grew confluently outside the growth inhibition zone, whereas the parental strains did not grow at all (Fig. 1A). The parental strains, however, began growing when the plate was incubated longer (Fig. 1B). It was noted that Hg^{2+} formed a much more sharply defined growth inhibition zone than did other heavy metals and that, unlike many other heavy-metal-resistant mutants, the mutants we isolated were not well distinguished from the parental strains by the final size of the growth inhibition zone.

Genetic characterization of mutants. Each mutant was crossed to a wild-type strain of opposite mating type (EH1-4A or EH1-4B). The resultant diploids were like the mutants in response to Hg^{2+}. Therefore, we conclude that the mutations conferring Hg^{2+} resistance are dominant. These diploids were sporulated, and the tetrads were analyzed (Table 1). All but one of the mutants gave rise exclusively to tetrads containing two Hg^{2+}-resistant segregants, which indicated that the corresponding mutants contained single mutations. Judging from the segregation pattern, we conclude that the exceptional mutant, R4A-1, carried two centromere-linked mutations.

TABLE 1. Segregation of the response to Hg^{2+}

Mutant[a]	Parental strain	Mutagen	No. of tetrads showing segregation of:[b]		
			2:2	3:1	4:0
R4A-1	EH1-4A	UV	12	2	10
R4A-2	EH1-4A	UV	8	0	0
R4A-3	EH1-4A	UV	12	0	0
R4A-4	EH1-4A	UV	10	0	0
RC-1	EH1-4B	None	12	0	0
RC-2	EH1-4B	None	10	0	0
R4B-1	EH1-4B	UV	13	0	0
R4B-2	EH1-4B	UV	10	0	0
R4B-3	EH1-4B	UV	10	0	0
R4B-4	EH1-4B	UV	20	0	0
RT-1	EH1-4B	UV	14	0	0
RF-1	EH1-4B	UV	12	0	0
RF-2	EH1-4B	UV	13	0	0
RF-3	EH1-4B	UV	14	0	0
RF-4	EH1-4B	UV	12	0	0
RF-5	EH1-4B	UV	11	0	0

[a] Mutants derived from strain EH1-4A were crossed to strain EH1-4B, and those from strain EH1-4B were crossed to strain EH1-4A.

[b] Ratios represent resistant (mutant) to normal (wild-type) responses to Hg^{2+}.

The mutation in R4B-4, designated *HGR1-1*, was used as a tester for linkage analysis. Eight mutants gave rise exclusively to resistant segregants when crossed to strains containing *HGR1-1* (Table 2), which indicated that mutations in these mutants were closely linked, and possibly allelic, to *HGR1-1*. The remaining eight mutants gave rise to both wild-type and resistant segregants, which indicated that they contained mutations unlinked to *HGR1-1*. The mutation in R4A-4, designated *HGR2-1*, was used for the second linkage analysis (Table 2). All mutants that gave rise to both wild-type and resistant segregants in the first analysis gave rise exclusively to resistant segregants in the second, which indicated that they contained mutations closely linked, and possibly allelic, to *HGR2-1* (Table 2). As expected, those that gave rise exclusively to resistant segregants in the first analysis, except for R4A-1, gave rise to both wild-type and resistant segregants in the second. The fact that R4A-1 produced no wild-type segregants in the second analysis indicates that it contained the second mutation close, or allelic, to *HGR2-1*. In short, all mutations we obtained in this study were linked, or allelic, to either *HGR1-1* or *HGR2-1*.

TABLE 2. Linkage analyses of Hg^{2+}-resistant mutations[a]

Mutant	Recombination with:		Mutant	Recombination with:	
	HGR1-1	*HGR2-1*		*HGR1-1*	*HGR2-1*
RC-2	−	+	R4A-2	+	−
R4B-1	−	+	R4A-3	+	−
R4B-4	−	+	R4A-4	+	−
RF-1	−	+	RC-1	+	−
RF-2	−	+	R4B-2	+	−
RF-4	−	+	R4B-3	+	−
RF-5	−	+	RT-1	+	−
R4A-1	−	−	RF-3	+	−

[a] Ten or more sets of tetrads were examined for each cross. R4B-4 and DH8-6B (*HGR1-1*) and R4A-4 and DH4-2B (*HGR2-1*) were used as testers. + and − represent the presence and absence, respectively, of recombinants (wild-type segregants).

TABLE 3. Mapping of *HGR1-1* on chromosome IV

Marker	Chromosome	Segregation[a]			Distance (centimorgans)
		P	N	T	
leu2	III	4	5	2	
trp1	IV	14[b]	0	2	6
pet17	XV	4	3	3	

[a] P, N, and T represent parental-ditype, nonparental-ditype, and tetratype tetrads, respectively.
[b] Statistically significant deviation from a parental-to-nonparental ratio of 1:1 at the 1% level.

HGR1-1 was mapped on chromosome IV because it showed linkage to *trp1-1* (Table 3).

Effects of *HGR1-1* and *HGR2-1* mutations on response to Hg^{2+}. Since strains containing both *HGR1-1* and *HGR2-1* were not distinguished from those containing *HGR1-1* or *HGR2-1* in terms of response to Hg^{2+}, we conclude that these mutations are not additive. Moreover, it was found that they did not confer resistance to methylmercury, phenylmercury, As^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, or Cd^{2+}. The 50% lethal dose of Hg^{2+} for the mutants was about 220 μM in YPD medium, which was not markedly different from that for the parental strains. The mutants, however, grew faster than the parental strains in the presence of sublethal doses of Hg^{2+} (Fig. 2A). The delayed growth of the parental strains was not due to induced resistance, because cells grown in the presence of Hg^{2+} showed the same delayed growth (Fig. 2B). This observation leads to the conclusion that Hg^{2+} resistance of the mutants we isolated was not due to constitutive expression of a usually repressed resistant mechanism.

The 50% lethal dose of Hg^{2+} in a synthetic medium was about 10 μM for both the parental strains and the mutants. Apparently, Hg^{2+} toxicity was about 20 times higher in the synthetic medium than in YPD medium. We therefore suspect that some substance(s) in YPD antagonized Hg^{2+} toxicity. More interestingly, we found that the parental strains and mutants responded to Hg^{2+} similarly in the synthetic medium. Therefore, it is likely that some substance(s) in YPD medium was responsible for the difference in the response to Hg^{2+}. At the moment, we do not know what the substance is.

Binding of Hg^{2+} to the cell wall. Since certain mercury-resistant strains of bacteria are known to detoxify Hg^{2+} by reducing it to volatile Hg^0 (14, 19, 20), we examined mercury volatilization in strains EH1-4A, EH1-4B, R4A-4, and R4B-4. Cells were inoculated in YPD medium containing 220 μM $^{203}Hg^{2+}$ (360 mCi/mol), and radioactivity of the culture was measured at various intervals. For all strains, radioactivity decreased at the same rate (about 5% of the initial radioactivity per 12 h) from the lag phase through the late stationary phase. Moreover, it was found that YPD medium alone volatilized Hg^{2+} at about the same rate. From this result, we conclude that Hg^{2+} resistance of the mutants is not attributable to enhancement of mercury volatilization.

Since resistance is often mediated by the reduced uptake of toxins, we next examined uptake of Hg^{2+} by cells (see Materials and Methods). It was found that EH1-4B took up Hg^{2+} linearly for at least 10 min. However, the cell-associated Hg^{2+} was released to some extent by EDTA and more effectively by cysteine and glutathionine (Fig. 3). This result indicates that Hg^{2+} binds to SH groups at or near the surface of the cell, presumably at the cell wall. When we examined strains EH1-4A, EH1-4B, R4A-4, and R4B-4, we found that the Hg^{2+}-resistant mutants (R4A-4 and R4B-4) took up Hg^{2+} as much as, or even slightly more than, the parental strains did (Table 4). However, when the cell wall was removed, the spheroplasts of the mutants and the parental strains retained 5 and 15%, respectively, of the cell-associated Hg^{2+}. That is, the spheroplast-associated Hg^{2+} was significantly less in the mutants than in the parental strains. These results indicate not only that the cell wall acts as a permeation barrier for Hg^{2+} but also that *HGR1-1* and *HGR2-1* confer Hg^{2+} resistance by increasing the Hg^{2+}-binding capacity of the cell wall.

Binding of Hg^{2+} to spheroplasts. The intact cells and the spheroplasts of strain EH1-4B were compared for uptake of Hg^{2+} (Fig. 4). Although the intact cells took up Hg^{2+} for the

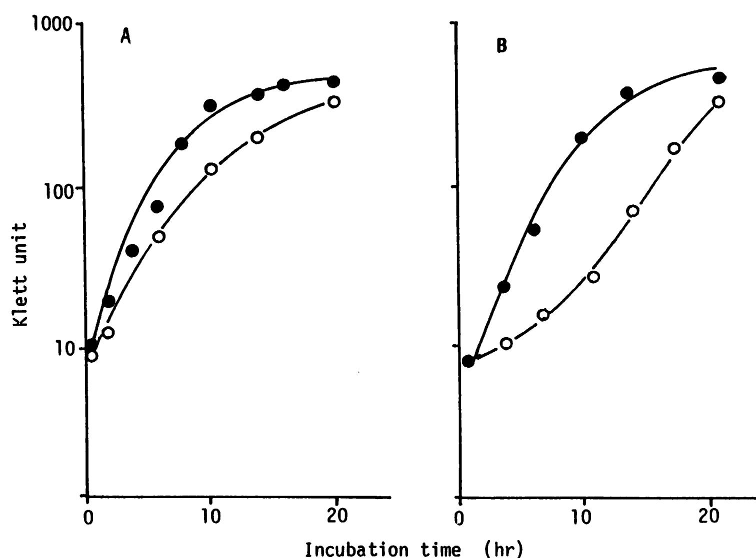

FIG. 2. Growth curve. Strain EH1-4B (○) and mutant R4B-4 (●) were grown overnight at 30°C in YPD medium and then inoculated in YPD medium containing 220 μM $HgCl_2$ (A). After 20 h of incubation, portions of the cultures were inoculated in fresh YPD medium containing 220 μM $HgCl_2$ (B).

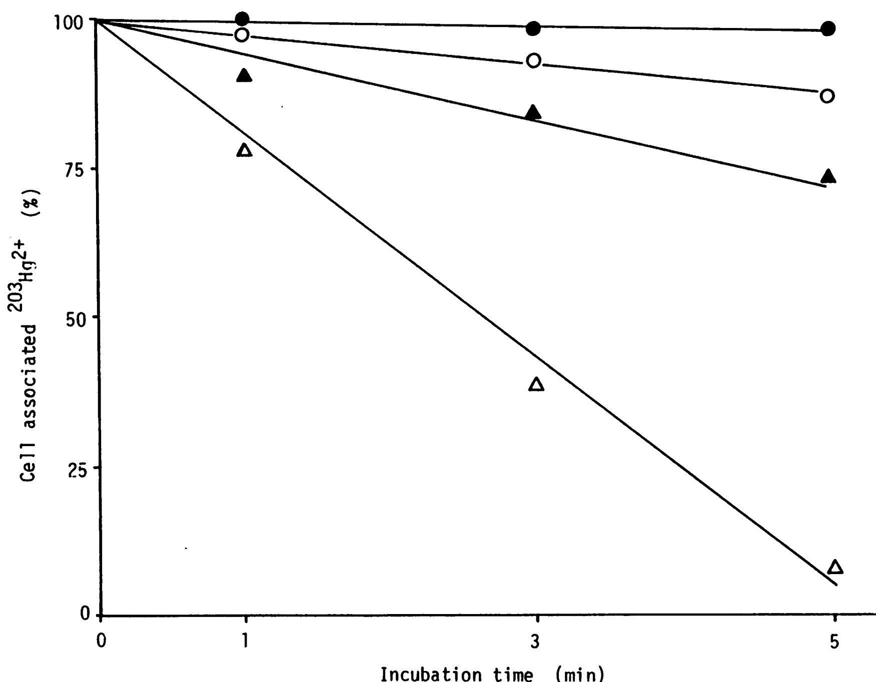

FIG. 3. Release of cell-associated Hg^{2+}. Cells treated with 220 μM Hg^{2+} for 10 min were thoroughly washed and then suspended in 0.1 M sodium phosphate buffer, pH 5.7 (●), or the same buffer containing 2.2 mM EDTA (○), cysteine (▲), or glutathione (△). The cells were harvested at various intervals, and radioactivity was measured.

entire period examined, the spheroplasts took up Hg^{2+} in only a very short period (less than 1 min). Therefore, after more than 10 min of incubation, significantly more Hg^{2+} was taken up by the cells than by the spheroplasts. This result suggests that Hg^{2+} does not permeate into the cell membrane well and that Hg^{2+} binds only at the surface of the cell membrane. Obviously, the cell membrane has fewer binding sites than does the cell wall. It is also evident that the existence of the cell wall reduced binding of Hg^{2+} to the cell membrane by about fivefold, supporting our argument that the cell wall acts as a permeation barrier for Hg^{2+}.

DISCUSSION

Murray and Kirby (8) have previously shown that a large portion of mercury in yeast cells grown in the presence of Hg^{2+} localizes in the cell wall fraction. Our finding that a large portion (85% or more) of the cell-associated Hg^{2+} localizes in the cell wall (Table 4) is in agreement with their observation. Since cell-associated Hg^{2+} is readily released upon treatment with SH compounds (Fig. 3), we conclude that Hg^{2+} binds to the cell wall mainly via SH groups; this conclusion is in accord with the previous notion that Hg^{2+} interacts most effectively with SH groups (16). Interestingly, however, binding of Hg^{2+} to the cell wall proceeds much slower than one might think (Fig. 4). The slow reaction is taken as an indication that the cell wall is a porous entity within which many Hg^{2+}-binding sites are embedded. In other words, the cell wall acts like an adsorbent filter. Hg^{2+} binds to the cell wall while it diffuses through it. Although we do not know which cell wall component(s) binds Hg^{2+}, we think that mannoproteins are the most likely candidates.

TABLE 4. Uptake of Hg^{2+}

Strain	Specific radioactivity[a]		B/A
	Cells (A)	Spheroplasts (B)	
EH1-4B	1,590 ± 390	221 ± 20	0.14
EH1-4A	1,630 ± 420	292 ± 11	0.18
R4B-4	1,890 ± 510	120 ± 7	0.06
R4A-4	2,440 ± 670	80 ± 4	0.03

[a] Cells were treated with $^{203}Hg^{2+}$ for 10 min, and cell-associated radioactivity was measured (see Materials and Methods). Spheroplasts were prepared from the Hg^{2+}-treated cells. Each experiment was repeated three times; data shown are averages and standard deviations, expressed as disintegrations per minute per 10^6 cells or spheroplasts.

Barkay and Colwell (1) have reported a mercury-resistant strain of *Pseudomonas fluorescens* which takes up more mercury than do other strains. Although this strain carries a mercury-resistant plasmid, it does not volatilize mercury well. Moreover, Hg^{2+} taken up by this mutant localizes exclusively in the cell envelope fraction. In short, the strain appears to be a cell wall mutant. Therefore, it is unquestionable that the cell wall is involved in the defense against Hg^{2+} in this organism. On the basis of the high reactivity of Hg^{2+} against SH groups, it is not surprising that the cell wall has such a function. It is of interest to know the extent to which the cell wall is involved in protection against Hg^{2+} in organisms such as bacteria, fungi, algae, and higher plants.

The cell membrane appears to have fewer Hg^{2+}-binding sites than does the cell wall (Fig. 4). However, this does not mean that binding of Hg^{2+} to the cell membrane is biologically less significant. Rather, we think that Hg^{2+} imposes some fatal damage on the cell by interacting with the cell membrane, since the cell membrane carries out much more vital functions than does the cell wall. In fact, we have already shown that Hg^{2+} inhibits tyrosine uptake, a function associated with the cell membrane (11). When we consider the biological effects of Hg^{2+}, especially at low concentra-

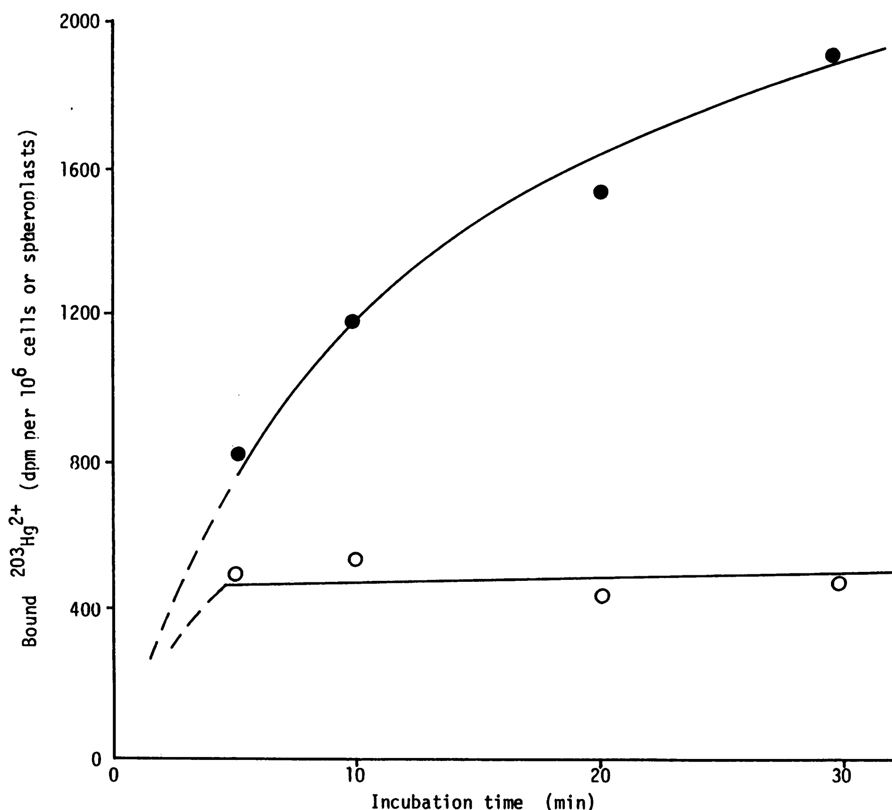

FIG. 4. Comparison of Hg^{2+} binding in cells and spheroplasts. Cells or spheroplasts of strain EH1-4B were suspended in an appropriate medium (see Materials and Methods) containing 17.4 nM Hg^{2+} (350 Ci/mol). Radioactivity taken up by the cells (●) or the spheroplasts (○) was measured at various intervals.

tions, we need to pay increased attention to the interaction of Hg^{2+} with the cell membrane.

It has been widely accepted that metallothioneins play a role in the defense against heavy metals, including mercury, in various organisms (7). In *S. cerevisiae*, a metallothionein that is responsible for Cu^{2+} resistance has been most well characterized; this metallothionein is enhanced by *CUP1* mutations (2–4, 21). Although it has been recently shown that Cd^{2+} resistance of a certain strain is attributable to a Cd^{2+}-binding protein(s) (6), the relationship between this protein and the *CUP1* metallothionein is not known. We should stress that the *CUP1* mutations do not confer Hg^{2+} resistance (B. Ono, unpublished data). Therefore, we contend that the *CUP1* metallothionein does not play a significant role in Hg^{2+} resistance. At the same time, we do not think that the cell wall plays a significant role in the defense against Cu^{2+} because the Hg^{2+}-resistant mutations discussed in this paper do not confer Cu^{2+} resistance. In short, it appears that the cell wall and the *CUP1* metallothionein are responsible for the defense against different heavy metals. We do not mean to imply that *S. cerevisiae* does not have an Hg^{2+}-thionein. Rather, we suspect that Hg^{2+} resistance acquired by training is mediated by a metallothionein. Of course, this has to be proven. With such a study, we will be able to develop a better understanding of Hg^{2+} resistance in *S. cerevisiae*.

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