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Role of Hydrosulfide Ions (HS^-) in Methylmercury Resistance in *Saccharomyces cerevisiae*

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Methylmercury-resistant mutants were obtained from *Saccharomyces cerevisiae*. They were divided into two complementation groups, *met2* (homoserine *O*-acetyltransferase deficiency) and *met15* (enzyme deficiency unknown), as reported previously. It was found that *met15* was allelic to *met17* (*O*-acetylserine and *O*-acetylhomoserine sulfhydrylase deficiency). Methylmercury toxicity was counteracted by exogenously added HS^-, and both *met2* and *met17* (*met15*) mutants overproduced H_2S. On the basis of these results, we conclude that *met2* and *met17* (*met15*) cause accumulation of hydrosulfide ions in the cell and that the increased level of hydrosulfide is responsible for detoxification of methylmercury.

Mercury is one of the environmental pollutants of most concern because it is highly toxic to living organisms and widely used in industry (4, 6, 10). In studying mercury toxicity, knowledge of the effects of mercury at cellular and subcellular levels is indispensable. For such studies use of model organisms is of importance, and *Saccharomyces cerevisiae* is appropriate in this respect because it is a unicellular eucaryotic organism, readily manipulated genetically, and easily analyzed biochemically.

Ono et al. (13) showed that Hg^{2+} resistance mutations were dominant and were divided into two groups by their linkage relationships. It was also shown that the cell wall acted as an adsorption filter for Hg^{2+} and that the Hg^{2+}-resistant mutants had the cell wall with increased Hg^{2+}-binding capacity. On the other hand, Singh and Sherman (20, 21) isolated methylmercury-resistant mutants and found that the mutants were methionine dependent. The responsible mutations were recessive and were divided into two complementation groups. Although one was identified as *met2*, which confers deficiency of homoserine *O*-acetyltransferase (EC 2.3.1.31) (3), the other did not correspond to any of the *met* mutations known at that time and was named *met15*. Whether the *met2* and *met15* mutations confer methylmercury resistance by the same mechanism remains unknown. To elucidate the biochemical nature of *met15* and the mechanism of methylmercury resistance, we isolated methylmercury-resistant mutants and characterized them genetically and biochemically. Here, we present the results of our work.

MATERIALS AND METHODS

Yeast strains and growth conditions. The yeast strains used in this study are listed in Table 1. Standard yeast growth media were used (11, 18). YPD medium contained 2% glucose, 2% peptone, and 1% yeast extract. The synthetic minimal (SD) medium was described by Wickerham (22). For solid media, 2% agar was added. The growth temperature was 30°C.

Genetic procedures. Methylmercury-resistant mutants were isolated as described by Singh and Sherman (20, 21) with minor modifications. Cells were spread on SD agar plates, and a filter paper disc (5 mm in diameter) was placed at the center of each plate. Methylmercuric chloride (5 to 10 μl of a 12 mM solution) was applied onto the discs, and then the plates were incubated for 5 to 7 days.

Complementation tests were achieved by mass mating. Strains to be tested were mixed with tester strains on YPD agar medium. The plates were incubated overnight, and the cells were transferred to selective plates (SD medium). The plates were incubated for 4 days, and then the growth was scored.

Growth kinetics. An aliquot of the overnight culture was added to fresh YPD liquid medium; the initial cell density was adjusted to approximately 10^4 cells ml^{-1}. Methylmercury, NaSH, and/or homoserine was added to the growth medium at the desired concentration. Growth was monitored by measuring the increase of the optical density at 600 nm with an automatic cell growth analyzer (Bioscreen C; Labsystems Japan Co., Ltd., Tokyo, Japan).

Measurement of H_2S production. Cells were inoculated on LA agar medium (0.3% peptone, 0.5% yeast extract, 4% glucose, 0.02% ammonium sulfate, 0.1% lead acetate, and 2% agar) and were incubated for a week at 30°C. On this plate, H_2S-producing colonies develop dark-brown color due to formation of PbS.

A more quantitative analysis was made as follows. Cells were grown overnight in YPD liquid medium, harvested, washed twice with water, and then suspended in SD liquid medium (360 ml) containing 30 μM methionine. N_2 gas (5 ml min^{-1}) was passed through the culture vessel and then through a trap containing 3.75 ml of 1% zinc acetate and 0.125 ml of 12% sodium hydroxide (5). The culture was placed in a rotary shaker (30 rpm). After cultivation, trapped H_2S was assayed by the method of Siegel (19). H_2S production (micromoles per cell per hour) was deduced by dividing the amount of trapped H_2S by the cumulative cell number and the duration of cultivation.

RESULTS

Mutant isolation. Methylmercury-resistant mutants were isolated as described in Materials and Methods. A clear zone of growth inhibition was formed around the filter paper disc containing methylmercury, but there were a few colonies

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TABLE 1. Strains used in this study

Strain	Genotype	Reference or source[a]
IS66-4C	*MATα*	Ono et al. (15)
SL381-8C	*MATa can1-100 cys1-72 lys1-1 met1-1 trp5-48*	S. Liebman (University of Illinois)
STX66-4A	*MATa ade2 gal2 lys4 met2 pha2 prt3 rad18 trp1*	YGSC
OK184-1C	MATα *cys1-3 cys3-1 met2*	This study
W109-18D	MATa *ade1 ade2 his2 his6 leu2 lys1 met3 trp5*	R. Rothstein (Columbia University)
IS483-1A	MATa *arg1 aro7-1 his3 leu2-1 lys4 met4 prt1 rad1 trp4 ura2*	This study
XS195-23B	MATa *ade5 arg4 his7 ilv3 leu2 lys7 met5 rad52-1 trp1*	YGSC
XJB3-1D	MATa *gal1 gal2 met6*	YGSC
S1896D	MATa *ade1 gal1 gal2 leu1 met7 pet trp1*	YGSC
OK29-2B	MATa *leu2-1 met8-1*	This study
XS209-11C	MATa *ade4 his3 leu2 met10 rad52-1 trp1 ura4*	YGSC
YAT56	MATa *aro2 cyh2 lys5 met13*	A. Toh-e (Tokyo University)
STX145-13D	MATa *cdc19 lys9 met14 rad4 trp1 tyr1 ura1*	YGSC
AS5-2D	MATa *met15-6 trp1-1*	Singh and Sherman (21)
S6	MATα *leu2-1 met15-6*	Singh and Sherman (21)
NA22-10B	MATa *met17*	This study
SF115-1A	MATα *met1-1*	This study

[a] YGSC, Yeast Genetic Stock Center, University of California, Berkeley.

within this region. They were picked, subcloned, and tested for methylmercury resistance by the filter disc method. We obtained five independent methylmercury-resistant mutants (MMR1 through MMR5) in this way.

Resistance levels of the mutants were quantitated by growth in YPD liquid medium containing various concentrations of methylmercury. A typical result is shown in Fig. 1.

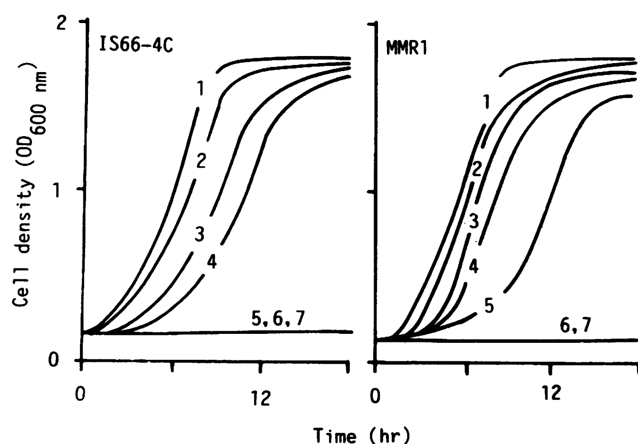

FIG. 1. Effect of methylmercury on growth of the parental strain (IS66-4C) and a methylmercury-resistant mutant (MMR1). Strains were grown overnight in YPD liquid medium, and portions of each culture were inoculated in YPD liquid medium containing the following concentrations of methylmercury: 1, 0 μM; 2, 0.5 μM; 3, 1.5 μM; 4, 2.5 μM; 5, 5.0 μM; 6, 7.5 μM; and 7, 10 μM. The cultures were incubated at 30°C, and the growth was monitored by measuring the increase of the optical density at 600 nm ($OD_{600\ nm}$).

TABLE 2. Complementation of methionine dependence of methylmercury-resistant mutants

Strain	Complementation[a] with: SL381-8C (*met1*)[b]	STX66-4A (*met2*)	NA22-10B (*met17*)	AS5-2D (*met15*)	Locus assignment
MMR1	+	+	−	−	*MET17*
MMR2	+	+	−	−	*MET17*
MMR3	+	+	±	±	*MET25* (?)
MMR4	+	−	+	+	*MET2*
MMR5	+	−	+	+	*MET2*
OK184-1C (*met2*)	+	−	+	+	*MET2*
S6 (*met15*)	+	+	−	−	*MET17*

[a] Complementation was judged by the loss of methionine dependence; +, ±, and − indicate good, weak, and no growth on methionineless medium, respectively.

[b] W109-18D (*met3*), IS483-1A (*met4*), XS195-23B (*met5*), XJB3-1D (*met6*), S1896D (*met7*), OK29-2B (*met8*), XS209-11C (*met10*), YAT56 (*met13*), and STX145-13D (*met14*) showed the same complementation pattern.

While a mutant (MMR1) and the parental strain (IS66-4C) responded similarly to methylmercury up to 0.5 μM, the latter grew more slowly than the former at higher concentrations (1.5 to 5.0 μM); at 5.0 μM, the parental strain did not grow at all while the mutant grew up, even though the initiation of growth was somewhat delayed.

Complementation of methionine dependence. The five mutants were subjected to complementation tests using strains containing the defined methionine mutations: *met1* through *met8*, *met10*, *met13* through *met15*, and *met17* (Table 2). Two mutants, MMR1 and MMR2, did not complement strains containing *met15* or *met17*. This result indicates either that the mutants contain both *met15* and *met17* or that *met15* and *met17* are alleles of the same gene. Since authentic *met15* and *met17* strains did not complement each other (Table 2), we conclude that the latter is the case. We hereafter refer to the mutation as *met17* or *met17* (*met15*) because the enzymatic deficiency has been defined under the name of *met17*; *met17* causes deficiency of a single protein which acts as *O*-acetylserine sulfhydrylase (EC 4.2.99.8) and *O*-acetylhomoserine sulfhydrylase (EC 4.2.99.10) (8, 9, 23). Though mutant MMR3 complemented all strains tested, its complementation with the *met17* strain was distinguishably weak. Since *met25*, which is shown to be allelic to *met17* by the gene-cloning studies (2, 17), has been reported to complement *met17* (8), we assume that MMR3 contains a *met25*-like mutation; unfortunately, a complementation test using an authentic *met25* strain was not conducted because such a strain was not available to us. The two remaining mutants, MMR4 and MMR5, did not complement the *met2* strain, suggesting that they contain mutations allelic to *met2*.

Effect of HS^- on methylmercury toxicity. From the S-amino acid biosynthetic pathway of *S. cerevisiae* (15), we suspected that *met2* and *met17* cause accumulation of homoserine and HS^- and that either or both of these compounds reduce the toxicity of methylmercury. We therefore examined the effect of these compounds added exogenously. Strain IS66-4C was inoculated in YPD liquid medium containing 5.0 μM methylmercury and various concentrations of homoserine or NaSH, and the growth was monitored (Fig. 2). NaSH dose-dependently counteracted methylmercury toxicity up to 0.1 μM. The antagonistic effect of NaSH saturated or tended to decrease at higher concentrations. We attribute this to the toxicity of NaSH itself, because NaSH

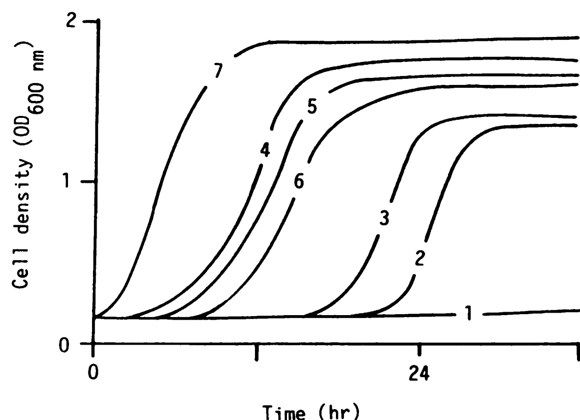

FIG. 2. Effect of NaSH on methylmercury toxicity. The parent strain (IS66-4C) was grown overnight in YPD liquid medium, and portions of the culture were inoculated in YPD liquid medium containing 5.0 μM methylmercury and the following concentrations of NaSH: 1, 0 μM; 2, 0.005 μM; 3, 0.01 μM; 4, 0.1 μM; 5, 0.5 μM; and 6, 50 μM. As a control, cells were grown in medium without methylmercury and NaSH (curve 7). Growth was monitored by measuring the increase of the optical density at 600 nm ($OD_{600\ nm}$).

added at these concentrations without methylmercury markedly inhibited growth (data not shown). In contrast, homoserine (up to 540 μM) did not affect the toxicity of methylmercury (data not shown). The results clearly indicate that HS^- but not homoserine counteracts the toxicity of methylmercury.

Production of H_2S by methylmercury-resistant mutants. Methylmercury-resistant mutants MMR1 (*met17*) and MMR4 (*met2*) and their parental strain (IS66-4C) were examined for H_2S production; a *met1* mutant (SF115-1A) defective for sulfate reduction (7) was included as a negative control (Fig. 3). While MMR1 and MMR4 yielded dark-brown colonies on LA agar medium, IS66-4C and SF115-1A did not. It was further found that MMR1 and MMR4 produced substantial amounts of H_2S while IA66-4C and SF115-1A did not (Table 3).

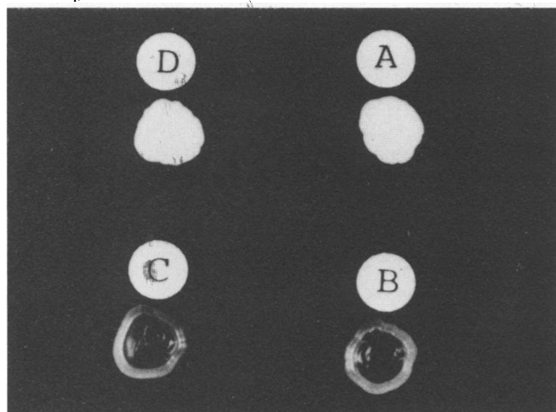

FIG. 3. Plate test of H_2S production. Cells were inoculated on LA agar plates and incubated at 30°C for 10 days. The following strains were tested: IS66-4C (wild type) (A), MMR4 (*met2*) (B), MMR1 (*met17*) (C), and SF115-1A (*met1-1*) (D).

TABLE 3. Production of H_2S

Strain	H_2S production (μmol cell^{-1} h^{-1} [10^{14}])[a]
IS66-4C (wild type)	<1.3
SF115-1A (*met1-1*)	<1.3
MMR1 (*met17*)	49 ± 25
MMR4 (*met2*)	42 ± 17

[a] Values represent the average and standard deviation obtained from three independent experiments for each strain.

DISCUSSION

We have shown in this study that both *met2* and *met17* confer methylmercury resistance via increased accumulation of H_2S. As far as we tested, the methylmercury-resistant mutants were only slightly resistant to Hg^{2+}, and the Hg^{2+}-resistant mutants (13) were not resistant to methylmercury at a detectable level (data not shown). Therefore, we conclude that methylmercury and Hg^{2+} resistances are different. Hg^{2+} reacts effectively with sulfhydryl (-SH) groups (1), and presumably because of this property it massively binds to the cell wall and the cell membrane (13) and inhibits their functions, such as transport. Tyrosine transport (14) and cysteine transport (12) are severely inhibited by Hg^{2+}. Since Hg^{2+} hardly penetrates into the cell (13), the cell must have developed in the course of evolution a protective aid on the cell surface (i.e., cell wall). In contrast, methylmercury because of its hydrophobicity appears to penetrate readily into the cell and causes damage in the cell. Although exogenously added HS^- reduced the toxicity of Hg^{2+}, the strains carrying *met2* or *met17* were not markedly resistant to Hg^{2+} (data not shown). Thus, we conclude that methylmercury is mainly detoxified by an increased level of HS^- in the cell.

Although we have presented evidence that the cell detoxifies methylmercury by using HS^-, we still do not know what chemical reaction contributes most in this phenomenon. Since it is known that H_2S aids the volatilization of methylmercury (16), removal of methylmercury from the cell via such a mechanism is possible. However, it is also possible that methylmercury is converted to Hg^{2+} in the cell and then reacts with HS^- to yield highly insoluble and less-toxic HgS. Studies of the chemical transformation of mercury in the cell and the involvement of HS^- in it are needed for a better understanding of the molecular basis of methylmercury resistance.

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REFERENCES

1. **Berlin, M.** 1979. Mercury [toxicity], p. 503–530. *In* L. Friberg, G. F. Nordberg, and V. B. Vouk (ed.), Handbook on the toxicology of metals. Elsevier/North Holland Biomedical Press, Amsterdam.
2. **D'Andrea, R., Y. Surdin-Kerjan, G. Pure, and H. Cerest.** 1987. Molecular genetics of *met17* and *met25* mutants of *Saccharomyces cerevisiae*: intragenic complementation between mutants of a single structural gene. Mol. Gen. Genet. **207:**165–170.
3. **de Robichon-Szulmajster, H., and H. Cherest.** 1967. Regulation of homoserine O-transacetylase, first step in methionine biosynthesis in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **28:**256–262.
4. **D'Itri, F. M.** 1972. The environmental mercury problem. CRC

Press, Inc., Boca Roton, Fla.

5. **Dreyfuss, J., and K. J. Monty.** 1963. The biochemical characterization of cysteine-requiring mutants of *Salmonella typhimurium*. J. Biol. Chem. **238**:1019–1024.
6. **Gavis, J., and J. F. Ferguson.** 1972. Cycling of mercury through the environment. Water Res. **6**:989–1008.
7. **Jones, E. W., and G. R. Fink.** 1982. Regulation of amino acid and nucleotide biosynthesis in yeast, p. 181–299. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*. Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Masselot, M., and H. de Robichon-Szulmajster.** 1975. Methionine biosynthesis in *Saccharomyces cerevisiae*. I. Genetical analysis of auxotrophic mutants. Mol. Gen. Genet. **139**:121–132.
9. **Masselot, M., and Y. Surdin-Kerjan.** 1977. Methionine biosynthesis in *Saccharomyces cerevisiae*. II. Gene-enzyme relationships in the sulfate assimilation pathway. Mol. Gen. Genet. **154**:23–40.
10. **Nishimura, H., and M. Kumagai.** 1983. Mercury pollution of fishes in Minamata Bay and surrounding water: analysis of pathway of mercury. Water Air Soil Pollut. **20**:401–411.
11. **Ono, B., Y. Ishino-Arao, T. Shirai, and S. Shinoda.** 1985. Genetic mapping of leucine-inserting UAA suppressors in *Saccharomyces cerevisiae*. Curr. Genet. **9**:197–203.
12. **Ono, B., and K. Naito.** The cysteine transport system of *Saccharomyces cerevisiae*. Yeast, in press.
13. **Ono, B., H. Ohue, and F. Ishihara.** 1988. Role of cell wall in *Saccharomyces cerevisiae* mutants resistant to Hg^{2+}. J. Bacteriol. **170**:5877–5882.
14. **Ono, B., E. Sakamoto, and K. Yamaguchi.** 1988. *Saccharomyces cerevisiae* strains sensitive to inorganic mercury. Curr. Genet. **11**:399–406.
15. **Ono, B., Y. Shirahige, A. Nanjoh, N. Andou, H. Ohue, and Y. Ishino-Arao.** 1988. Cysteine biosynthesis in *Saccharomyces cerevisiae*: mutation that confers cystathionine β-synthase deficiency. J. Bacteriol. **170**:5883–5889.
16. **Rowland, I. R., M. J. Davis, and P. Grasso.** 1977. Biosynthesis of methylmercury compounds by the intestinal flora of the rat. Arch. Environ. Health **32**:24–28.
17. **Sangsoda, S., H. Cherest, and Y. Surdin-Kerjan.** 1985. The expression of the *MET25* gene of *Saccharomyces cerevisiae* is regulated transcriptionally. Mol. Gen. Genet. **200**:407–414.
18. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1974. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Siegel, L. M.** 1965. A direct microdetermination of sulfide. Anal. Biochem. **11**:126–132.
20. **Singh, A., and F. Sherman.** 1974. Association of methionine requirement with methylmercury resistant mutants of yeast. Nature (London) **247**:227–229.
21. **Singh, A., and F. Sherman.** 1974. Characteristics and relationships of mercury-resistant mutants and methionine auxotrophs of yeast. J. Bacteriol. **118**:911–918.
22. **Wickerham, L. J.** 1956. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeast. J. Bacteriol. **52**:293–301.
23. **Yamagata, S., K. Takeshima, and N. Naiki.** 1975. O-acetylserine and O-acetylhomoserine sulfhydrylase of yeast; studies with methionine auxotrophs. J. Biochem. **77**:1029–1036.