

Fig. 11. *FPS1* 欠損酵母における *HOG1* 欠損が亜ヒ酸添加後の細胞内ヒ素蓄積量に与える影響

酵母(1×10^8 cells)を0.2mMの亜ヒ酸を含む液体培地で3時間培養後、湿式灰化し、ICP-MSにより細胞内のヒ素量を測定した(n=5)。WTの値を100%として表に示した。

ヒ素の取り込み速度

hog1 Δ : 379 $\mu\text{g}/\text{min}/10^{10}\text{cells}$

Wild type : 110 $\mu\text{g}/\text{min}/10^{10}\text{cells}$

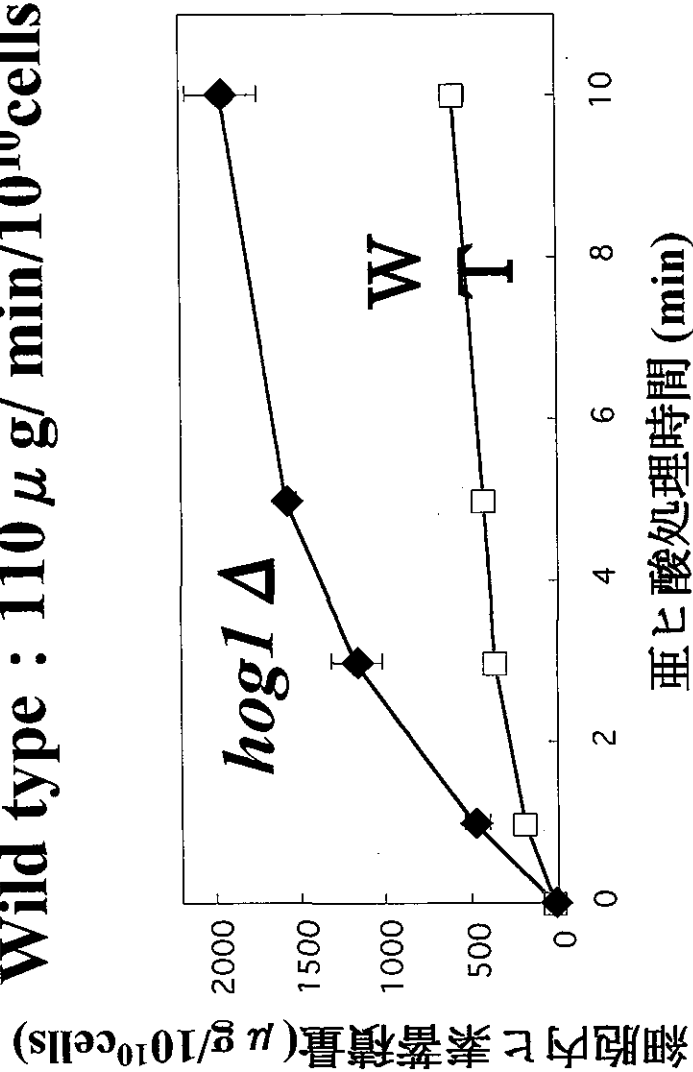


Fig. 12(a). *HOG1*欠損酵母のヒ素の取り込み速度

酵母($1 \times 10^8\text{cells}$)を0.2mMの亜ヒ酸を含む液体培地で培養後、湿式灰化し、ICP-MSにより細胞内のヒ素量を測定した($n=3$)。ヒ素の取り込み速度は0min, 1min, 3minの値を直線で取りその傾きを求めた。

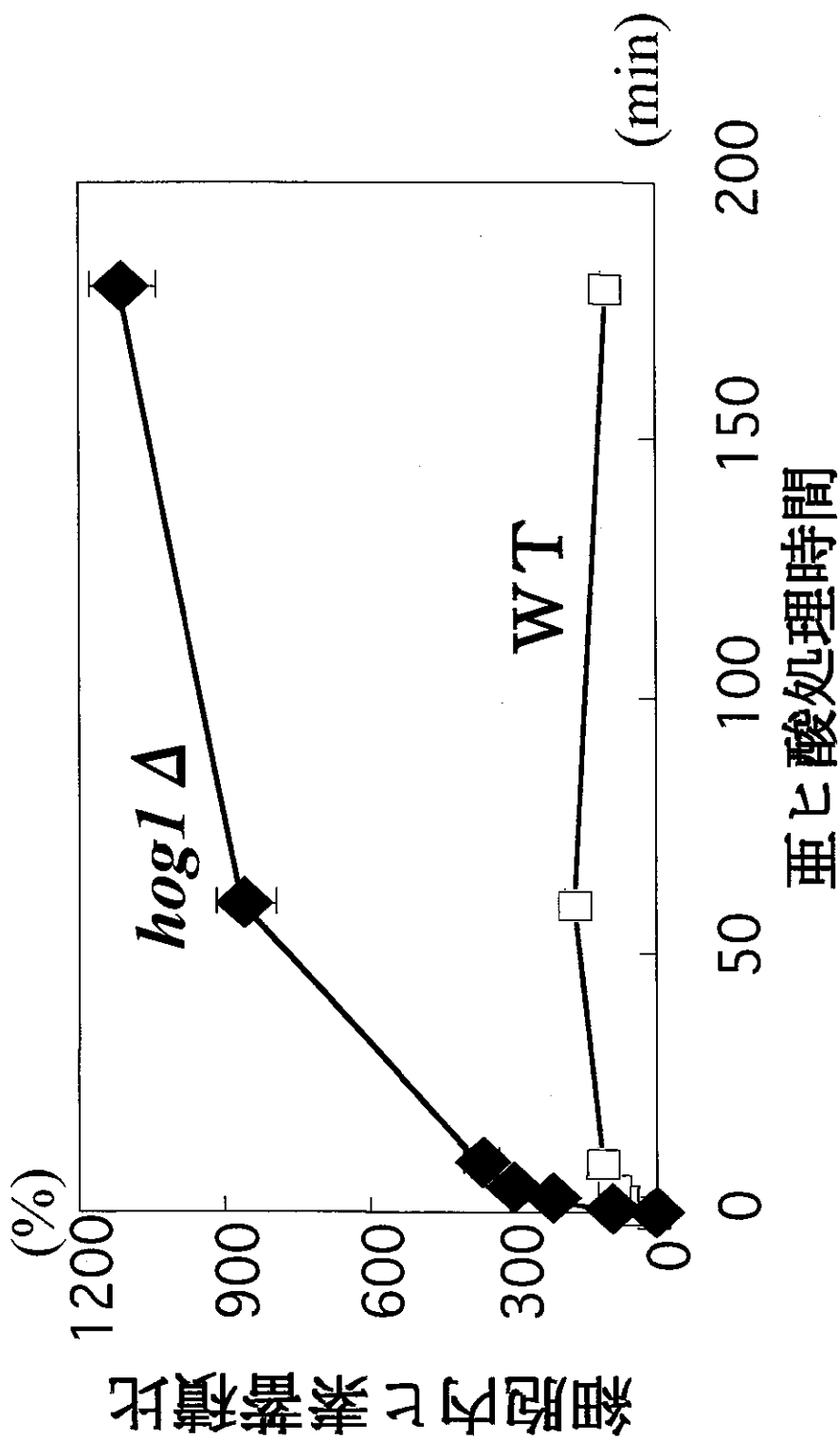
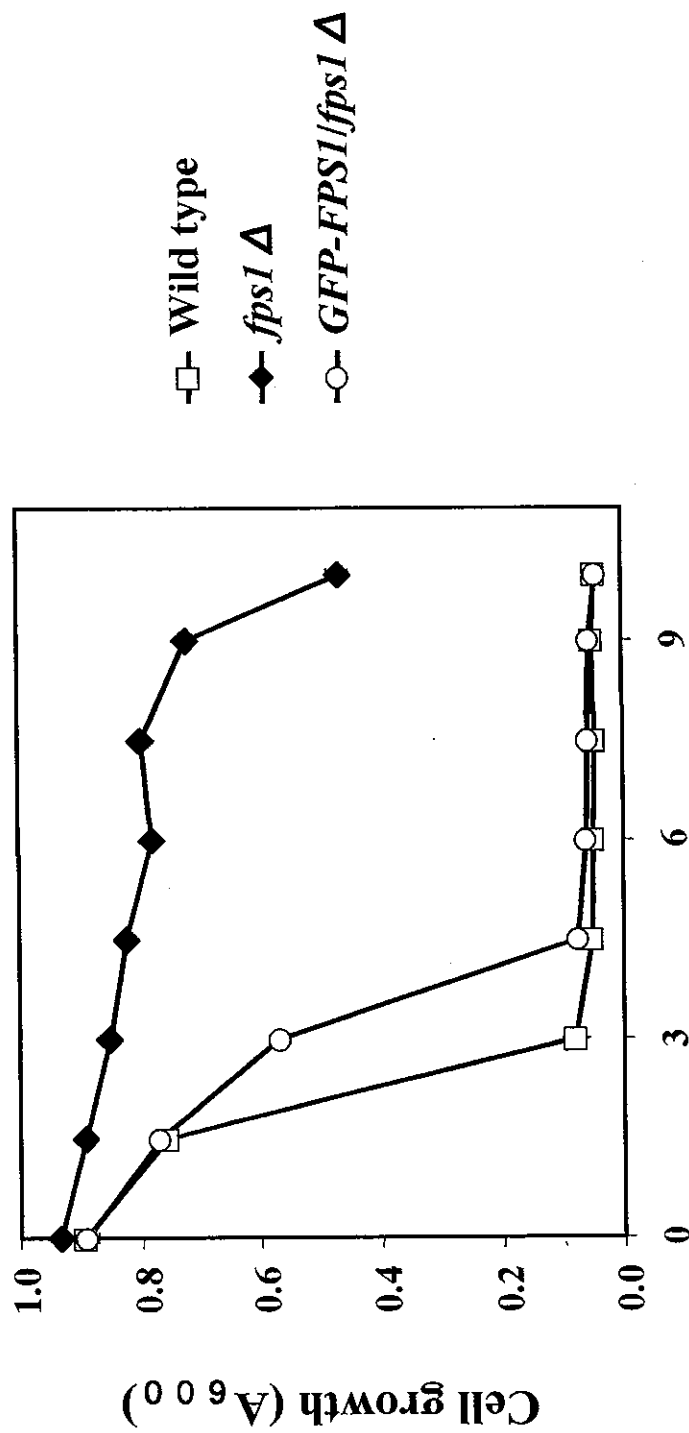


Fig. 12(b). *HOG1*欠損が亜硫酸添加後の細胞内ピルビン酸蓄積量に与える経時的な変化

酵母(1×10^8 cells)を0.2mMの亜硫酸を含む液体培地で培養後、湿式灰化し、ICP-MSにより細胞内のピルビン酸量を測定した(n=3)。WTにおける亜硫酸添加後180min後の値を100%として表に示した。



NaAsO₂(mM)

Fig. 13. *FPS1* 欠損酵母への *GFP-Fps1* 導入が亜ヒ酸感受性に与える影響

酵母を96 well plate 中で各濃度の亜ヒ酸存在下48時間培養した後、600 nmの吸光度を測定した(n=3)。

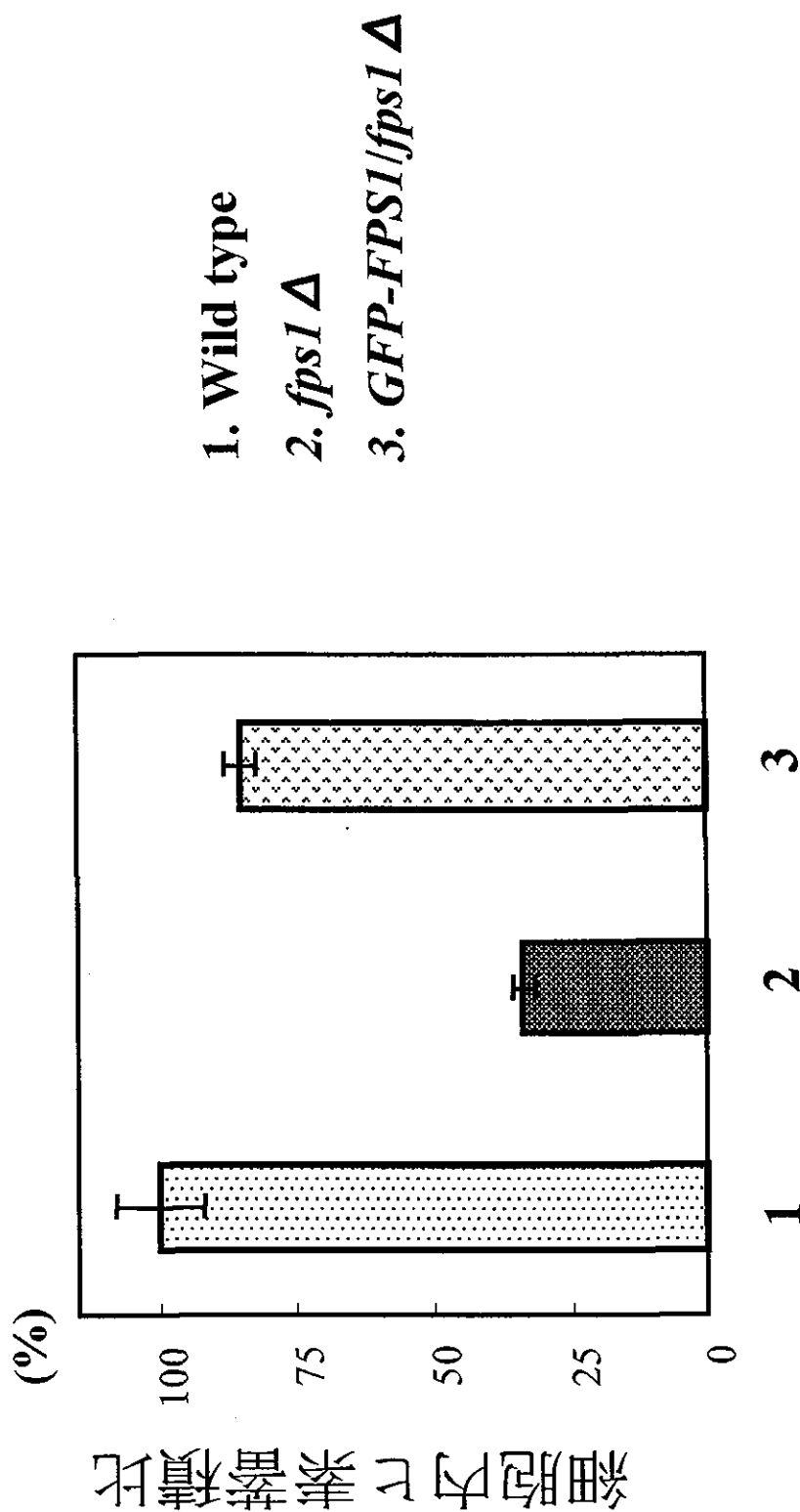
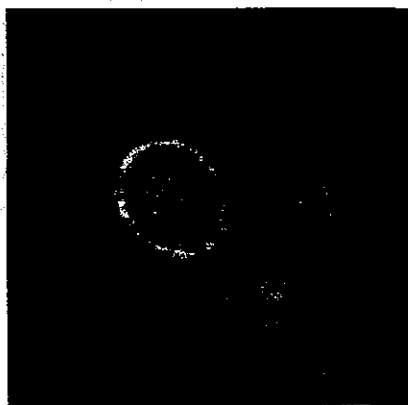


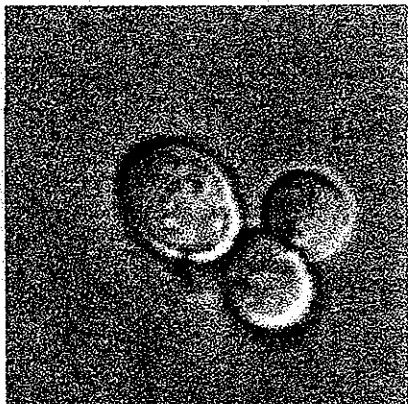
Fig. 14. *FPS1*欠損酵母へのGFP-Fps1導入が亜ヒ酸添加後の細胞内ヒ素蓄積量に与える影響

酵母(1×10^8 cells)を0.2mMの亜ヒ酸を含む液体培地で3時間培養後、湿式灰化し、ICP-MSにより細胞内のヒ素量を測定した(n=3)。WTの値を100%として表に示した。

GFP



透過像



FPS1/fps1 Δ

FPS1/fps1 Δ hog1 Δ

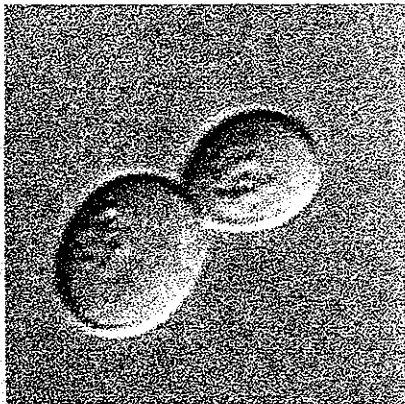
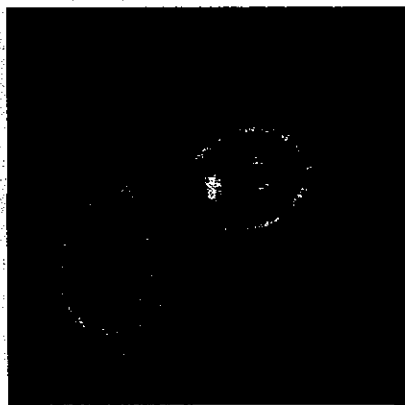
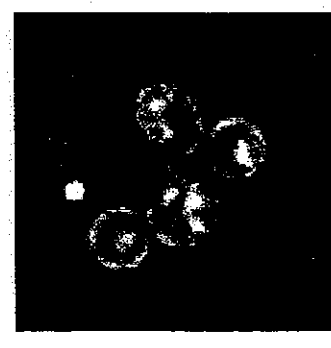


Fig. 15 HOG1欠損がFps1の細胞内分布に与える影響

酵母にFPS1-GFP発現プラスミドを導入した際のGFPの蛍光を検出することでFps1細胞内分布を検討した。

透過像

GFP



亜ヒ酸処理時間

0min

60min

Fig.16 亜ヒ酸処理がFps1の細胞内分布に与える影響

FPS1-GFP 発現プラスミドを導入した酵母に亜ヒ酸を処理した際のGFPの蛍光を検出することでFps1 細胞内分布を検討した。

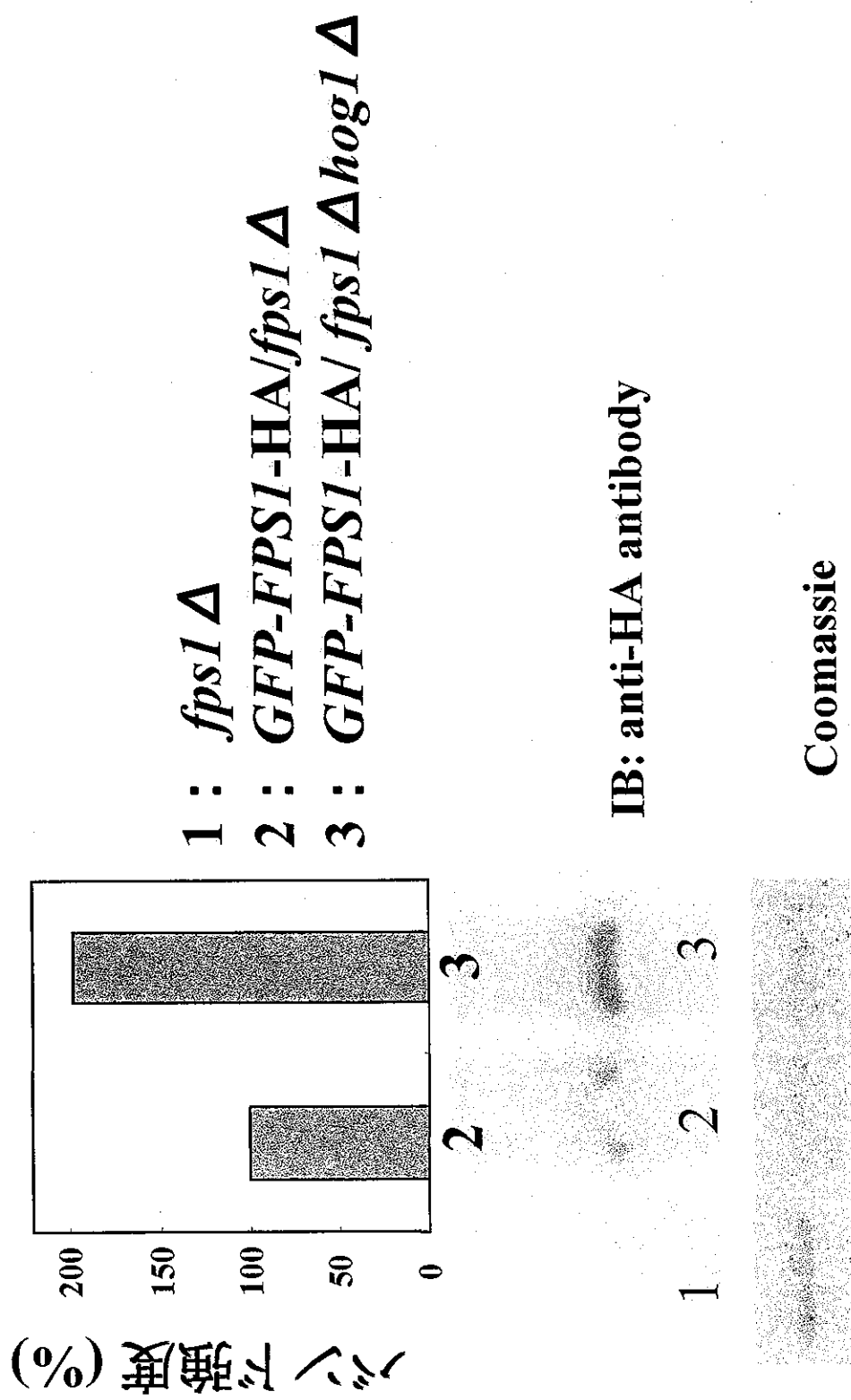


Fig. 17. 細胞膜中のFps1蛋白質量のHOG1欠損による変動

Fps1欠損酵母にFPS1-GFP-HA発現プラスミドを導入した株においてFps1-GFP-HA蛋白質のバンド強度を100%として表に示した。

別添 5 研究成果の刊行に関する一覧表

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

なし。

雑誌

発表者氏名	論文タイトル	発表雑誌名	巻号	ページ	出版年
Hwang, G. W. Furuoya, Y. Hiroshima, A. Furuchi, T. and Naganuma, A.	Overexpression of Bop3 confers resistance to methylmercury in <i>Saccharomyces cerevisiae</i> through interaction with other proteins such as Fkh1, Rts1 and Msn2	Biochem. Biophys. Res. Commun.	330	378-385	2005

IV. 研究成果の刊行物・別刷



Overexpression of Bop3 confers resistance to methylmercury in *Saccharomyces cerevisiae* through interaction with other proteins such as Fkh1, Rts1, and Msn2

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Abstract

We found that overexpression of Bop3, a protein of unknown function, confers resistance to methylmercury in *Saccharomyces cerevisiae*. Bmh2, Fkh1, and Rts1 are proteins that have been previously shown to bind Bop3 by the two-hybrid method. Overexpression of Bmh2 and the homologous protein Bmh1 confers resistance to methylmercury in yeast, but overexpression of either Fkh1 or Rts1 has a minimal effect. However, the increased level of resistance to methylmercury produced by overexpression of Bop3 was smaller in Fkh1-deleted yeast as compared with that of the wild-type strain. In contrast, the degree of resistance was significantly elevated in Rts1-deleted yeast. Msn2 and Msn4 were previously reported as proteins that bind to Bmh1 and Bmh2. Overexpression of Msn2 conferred a much greater sensitivity to methylmercury in yeast, while deletion of the corresponding gene lowered the degree of resistance to methylmercury induced by overexpression of Bop3. These results suggest that multiple proteins are involved in minimizing the toxicity of methylmercury induced by overexpression of Bop3.

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Keywords: Methylmercury; Resistance; Yeast; Bop3; Rts1; Fkh1

Methylmercury, the predominant form of organic mercury present in the environment, is a toxic compound producing severe disorders in the human central nervous system [1–3]. Fish are susceptible to the bioaccumulation of methylmercury, which may then be ingested by humans in their daily diet. In Japan in the 1950s thousands of people developed central nervous system disorders through eating large amounts of fish and shellfish polluted with methylmercury [4]. Recent epidemiological studies have indicated that ingestion

of methylmercury in fish during pregnancy can result in neurotoxicological effects in the offspring [5]. A warning was issued in several countries recommending a restriction of fish in the diet of pregnant women and infants. Given the severe nature of this poison, methods for both the prophylaxis and treatment of methylmercury intoxication are required. Unfortunately the mechanism underlying the toxicity of methylmercury is not fully understood. Furthermore, the nature of the protective mechanism that exists in some organisms has not been elucidated.

To elucidate the mechanism of methylmercury toxicity, we examined intracellular factors involved in yeast. Yeast is a genetically well-characterized eukaryotic

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organism that shares many genes common with mammals, including humans. Thus, the factors found in yeast are expected to function similarly in mammalian cells. We have already identified and reported two proteins, GFAT [6–8] and Cdc34 [8–10], which confer resistance to methylmercury in yeast. Genes encoding similar proteins have been found in humans. However, it is anticipated that many additional factors are involved in the development of methylmercury toxicity. In the present study, we have conducted further screening for proteins capable of conferring resistance to methylmercury in yeast and succeeded in identifying a new protein of unknown function, Bop3. Bop3 is a protein that was identified as a multicopy suppressor that could compensate for deletion of Pam1 [11], which was identified as a multicopy suppressor on the deletion of serine/threonine phosphatase 2A (PP2A) [12]. Although the function of Bop3 is unclear, extensive investigations conducted with the two-hybrid method demonstrated that Bop3 is capable of binding to Bmh2 [13], Fkh1 [14], and Rts1 [14]. Our results suggest that although Pam1 is not directly involved in resistance to methylmercury induced by overexpression of Bop3, these binding proteins and their homologues are involved, at least partially, in the resistance mechanism. The results of the present study indicate the involvement of Bop3 in the regulation of some important intracellular functions that exert protective effects against methylmercury toxicity through interaction with multiple proteins.

Materials and methods

Selection of genes that confer resistance to methylmercury. Methylmercury resistance genes were screened as described previously [9,10]. Briefly, *Saccharomyces cerevisiae* W303B (*MAT* α his3 can1-10 ade2 leu2 trp1 ura3) was transformed with a genomic DNA library that had been constructed by inserting a fragment of yeast chromosomal DNA into the *LEU2*-based multicopy plasmid Yep13 [10,15–17]. Transformants were grown in synthetic dextrose (SD) liquid medium without leucine (–Leu) at 30 °C. The Leu⁺ transformants were cultured (10⁵ cells/200 μ l per well) in SD (–Leu) medium for 24 h at 30 °C in the presence of a subtoxic concentration of methylmercury (0.4 μ M) in 96-well plates. Transformed cells that had proliferated rapidly and had formed aggregates in the presence of methylmercury were isolated from individual wells and plated on agar-solidified SD (–Leu) medium at a cell density of 5 \times 10³ cells per 10-cm plate. After incubation for 24 h at 30 °C, colonies were collected and their sensitivity to methylmercury was examined. The sensitivity of yeast cells from each colony was determined by culturing cells (10⁵ cells/200 μ l per well) in SD (–Leu) medium that contained various concentrations of methylmercury for 48 h. We chose eight colonies that exhibited strong resistance to methylmercury and isolated plasmids from them as described previously. The plasmids were amplified in *Escherichia coli* and then reintroduced into W303B cells to confirm the phenotype. We selected the plasmid that conferred the strongest resistance to methylmercury for further study. The nucleotide sequence of the genomic insert in the selected plasmid was determined with an automated DNA sequencer (Li-Cor, Lincoln, NE). After mapping, the genomic insert was excised and subcloned into the pRS425 vector. Subclones were

introduced into W303B yeast cells and the sensitivity to methylmercury of each resultant cell line was determined.

Quantification of growth inhibition by methylmercury and other compounds. The toxic effects of various agents on yeast cells were quantified during growth of cells in SD liquid medium (–Leu or –Ura). A suspension of cells (10⁶ cells per well) was grown in a 200 μ l aliquot of fresh liquid medium that contained one of the respective compounds at the concentration indicated. After incubation for 48 h, the absorbance at 620 nm (*A*₆₂₀) was measured spectrophotometrically as an index of cell growth.

Construction of gene expression vectors. The genes from yeast genomic DNA were amplified by PCR with the following oligonucleotides as primers:

5'-GACCAAAAACGGCGTTAAGA-3' and
5'-CCTTTATCCCATCGACAAAGC-3' for the *BOP1* gene;
5'-TCTCCGGGTAGAAGTGAAG-3' and
5'-TGCTAGTACAACACGTTTGG-3' for the *BOP2* gene;
5'-GTTGCTCTTCTAGTGCAGTTG-3' and
5'-TACATACGTAGGGGAACATC-3' for the *BOP3* gene;
5'-CCTACTTTTCAAATTGAGAG-3' and
5'-GAACTACAAATTATTACACCC-3' for the *BMH1* gene;
5'-CAAATCAACAAAAGTACCCG-3' and
5'-CTTCAATTTCCCTTGTATTTC-3' for the *BMH2* gene;
5'-GCTCATAGAAGAACTAGATC-3' and
5'-AGCCGTAAGTTCATAAGTC-3' for the *MSN2* gene;
5'-TAACGCCITTTATCAGTTCGGC-3' and
5'-TTGTCATACCGTAGCTTGTC-3' for the *MSN4* gene.

The resulting PCR products were ligated into the pGEM-T Easy or pTarget vector (Promega, Madison, WI). Each insert was digested with restriction endonucleases as follows: *KpnI* and *XhoI* for the *BOP1*, *BOP2*, and *BOP3* genes; *NotI* for the *MSN2* gene; and *EcoRI* for the *BMH1*, *BMH2*, and *MSN4* genes, and fragments were ligated into the pKTI0-GAPDH (*URA3*) yeast expression vector. Sequences of constructs were verified with an automated sequencer.

Gene disruption. Genes of the W303B yeast strain were disrupted as described previously [9,17]. For constructions of the *bmh1::HIS3*, *bmh2::HIS3*, *fkh1::HIS3*, and *rts1::HIS3* vector, the *HIS3* gene was amplified by PCR with the following oligonucleotides as primers: *BMH1*-HIS-F (5'-ATGTCAACCAGTCGTGAAGATTCTGTGTA CCTAGCCAAGTTGGCTGAACACTCTTGGCCTCCTCTAG-3') and *BMH1*-HIS-R (5'-TTACTTTGGTGCTTCACCTTCGGCGGC AGCAGGTGGCTGCTGTTGCTGATTTCGATTCAGAATGACAC G-3') for disruption of the *BMH1* gene; *BMH2*-HIS-F (5'-ATG TCCCAAACCTCGTGAAGATTCTGTTACCTAGCTAAATTAGC TGAACACTCTTGGCCTCCTCTAG-3') and *BMH2*-HIS-R (5'-TTATTGTTGGTTCACTTGATTTGTTTCAGCTGGAGCTT GTTGTGCTTCGTTTCAAGAATGACACG-3') for disruption of the *BMH2* gene; *MSN2*-HIS-F (5'-ATGACGGTCGACCATGATTC AATAGCGAAGATATTTTATTTCCCATAGACTCTTGGCCTCC TCTAG-3') and *MSN2*-HIS-R (5'-GTGATAAAATTAGTGTCATCA TCATCATCATTCAATAAGAGATCACTAGAATCGTTTCAGAA TGACACG-3') for disruption of the *MSN2* gene; *FKH1*-HIS-F (5'-ATGTCGTACCAGTAGGGAACAAAATTTAGTGGTAAAG ATAGTCGACTCTTGGCCTCCTCTAG-3') and *FKH1*-HIS-R (5'-ATTCCTCCTCGGTGTAATTTTCATCTTCTTCTTCTT CCATCATAAATTCGTTTCAAGAATGACACG-3') for disruption of the *FKH1* gene; *RTS1*-HIS-F (5'-ATGATGCGTGGTTTCAAGCA AAGATTAATAAAGAAGACCACCGGGTCTTCTTGGCCT CCTCTAG-3') and *RTS1*-HIS-R (5'-TCGAATCTAGATGAAGAA TGACGTTGGGGAGTCTTAATTAACCTAAATCTCGTTTCAG AATGACACG-3') for disruption of the *RTS1* gene. To disrupt the *PAM1* gene of W303B strain yeast, *pam1::KAN^r* was amplified from the *PAM1*-disrupted yeast (Complete Set of *S. cerevisiae* Gene Deletion Strains; Euroscarf, Frankfurt, Germany), which is a deletion

mutant of BY4742 strain yeast, by PCR with primers 5'-GGCGATTGCTTCCATATTCT-3' and 5'-GGCAACGTTTT CAATGGTCA-3'. The PCR products were introduced into yeast strain W303B to construct the corresponding disruptant. Disruption of the gene was verified by PCR analysis with primers:

5'-GGCATATCTCGGATAGGTAT-3' and
5'-TTGGATTGAAAGACAAGG-3' for the *PAM1* gene;
5'-GAACTACAAATTATTACACCC-3' and
5'-CAAATCAACAAAAAGTACCCG-3' for the *BMH1* gene or
5'-CTTCATTTCCCTTGTATTTTC-3' for the *BMH2* gene;
5'-GTCATAGAAGAAGACTAGATC-3' and
5'-AGCCGTAAGCTTCATAAGTC-3' for the *MSN2* gene;
5'-GCAAAGAAAGGCTTGGAGAGA-3' and
5'-ATACATATGGGTTCGACGACG-3' for the *FKH1* gene;
5'-CGTGCTATTTTCGAACATCCA-3' and
5'-TCCTCACTTCTCGAGCTTGT-3' for the *RTS1* gene.

Results and discussion

To identify genes whose overexpression confers resistance to methylmercury in *S. cerevisiae*, we introduced a library of yeast chromosomal DNA fragments in the 2- μ m multicopy vector Yep13, into the yeast strain W303B. Eight transformants were obtained that could grow on SD (-Leu) medium containing an otherwise lethal amount of methylmercury (0.4 μ M). The transformant P54, which exhibited the strongest resistance, was selected for further study. Partial sequencing of clone P54 and a subsequent search of the *Saccharomyces* Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>) revealed that the insert corresponded to region 544177–550829 on chromosome 14 of *S. cerevisiae*. Two open reading frames, *YIP3* and *BOP3*, were contained on the P54 fragment. Each open reading frame was excised from pRS425-P54 using appropriate restriction enzymes to give two fragments, F1 and F2 (Fig. 1A). These two fragments were subcloned into pRS425 vector. Sensitivity to methylmercury was then examined after introduction of the respective plasmids into the parent yeast strain. Yeast harboring pRS425-F1 (pRS425-BOP3) showed stronger resistance to methylmercury as compared to yeast harboring pRS425 or pRS425-F2 (Fig. 1B). These results indicated that *BOP3* is involved in resistance to methylmercury.

First, we examined the sensitivity of Bop3-overexpressing yeast cells to heavy metal compounds other than methylmercury. Yeast cells overexpressing Bop3 did not show resistance to mercury compounds, such as inorganic mercury and *p*-chloromercuribenzoic acid (pCMB), nor other heavy metals, such as cadmium and copper (data not shown). Thus, overexpression of Bop3 in yeast seemed to impart a relatively specific resistance to methylmercury.

Bop3 (Bypass Of Pam1) is a protein with unknown function that was identified, together with Bop1 and

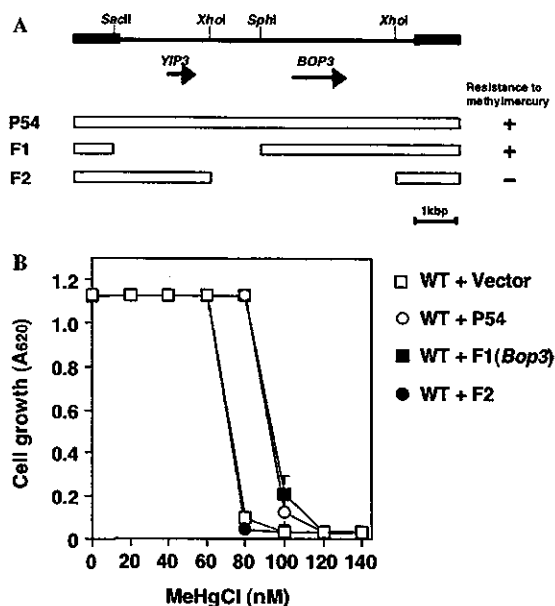


Fig. 1. Identification of a gene that confers resistance to methylmercury. (A) Restriction map of the genomic DNA insert (P54) that conferred resistance to methylmercury. The thick black lines represent the vector YEp13; the thin line represents the genomic DNA inserts. The restriction sites used to generate different subclones are indicated by vertical lines above the yeast genomic DNA insert (P54). The ability of two subclones (F1 and F2) to confer methylmercury resistance is indicated (+, conferred resistance; -, did not confer resistance). ORFs are indicated by black arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow. (B) Sensitivity to methylmercury of yeast that harbored plasmids with the indicated inserts. Yeast cells transformed with pRS425-P54, pRS425-F1, pRS425-F2 or pRS425 were cultured in SD (-Leu) liquid medium that contained methylmercuric chloride at the indicated concentration. After 48-h incubation the absorbance of the culture was measured at 620 nm. Each point represents the mean value of results from three cultures with SD (bars). The absence of a bar indicates that the SD falls within the symbol.

Bop2, as a multicopy suppressor of Pam1 [11]. Therefore, we examined the effect of overexpression of either Bop1 or Bop2 on the sensitivity to methylmercury. Overexpression of Bop1 or Bop2 in yeast resulted in a similar sensitivity to methylmercury as that of the control (i.e., yeast harboring the vector, pKT10) (Fig. 2A). These results indicate that the function as a multicopy suppressor of Pam1 is not needed for the protective effects of Bop3 against methylmercury.

Pam1 was identified as a multicopy suppressor on PP2A deletion [12], but the mechanism of action of this protein remains unclear. We generated a PAM1-deleted strain (*pam1* Δ) and examined its sensitivity to methylmercury. Almost no difference was noted with regard to the sensitivity to methylmercury of the *pam1* Δ strain in comparison to the control yeast (Fig. 2B). Sensitivity to methylmercury was also found to be nearly the same when Bop3 was overexpressed in the wild-type strain and *pam1* Δ strain, respectively (Fig. 2B). Thus, Pam1

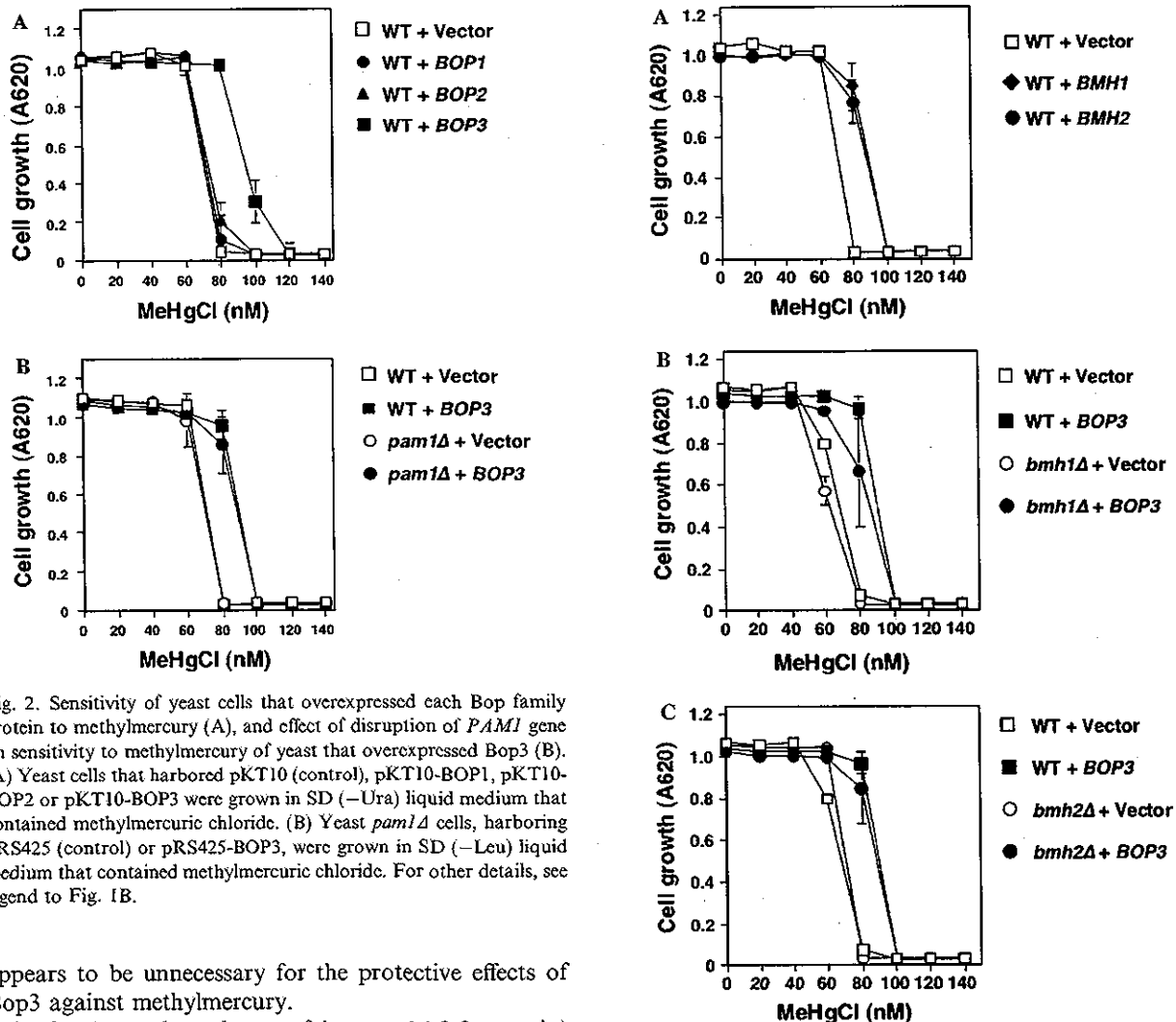


Fig. 2. Sensitivity of yeast cells that overexpressed each Bop family protein to methylmercury (A), and effect of disruption of *PAM1* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B). (A) Yeast cells that harbored pKT10 (control), pKT10-BOP1, pKT10-BOP2 or pKT10-BOP3 were grown in SD (-Ura) liquid medium that contained methylmercuric chloride. (B) Yeast *pam1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (-Leu) liquid medium that contained methylmercuric chloride. For other details, see legend to Fig. 1B.

appears to be unnecessary for the protective effects of Bop3 against methylmercury.

Bmh2 (yeast homologue of human 14-3-3 protein) [18,19], Fkh1 (transcription factor of forkhead type) [20], and Rts1 (subunit of protein phosphatase 2A) [21–23] have been identified as proteins that bind to Bop3. We next examined the possible role of these Bop3-binding proteins in the mechanism of acquisition of resistance to methylmercury by overexpression of Bop3. 14-3-3 is a protein conserved in most eukaryotes and is known to play a versatile role within the cell, such as regulation of apoptosis through binding to p53 and regulation of the cell cycle through binding to the Cdc2-CycB complex [24–26]. It was also reported that not only Bmh1 but also Bmh2, a homologous protein of Bmh1, was involved in RAS/MAPK cascade signaling in yeast [18,27]. We examined the effects of overexpression of Bmh1 and Bmh2 in yeast on the sensitivity to methylmercury. Yeast cells that overexpress Bmh1 or Bmh2 showed a significant resistance to methylmercury compared with the control harboring only the vector (Fig. 3A). However, there were differences with regard to the degree of resistance among the clones over-

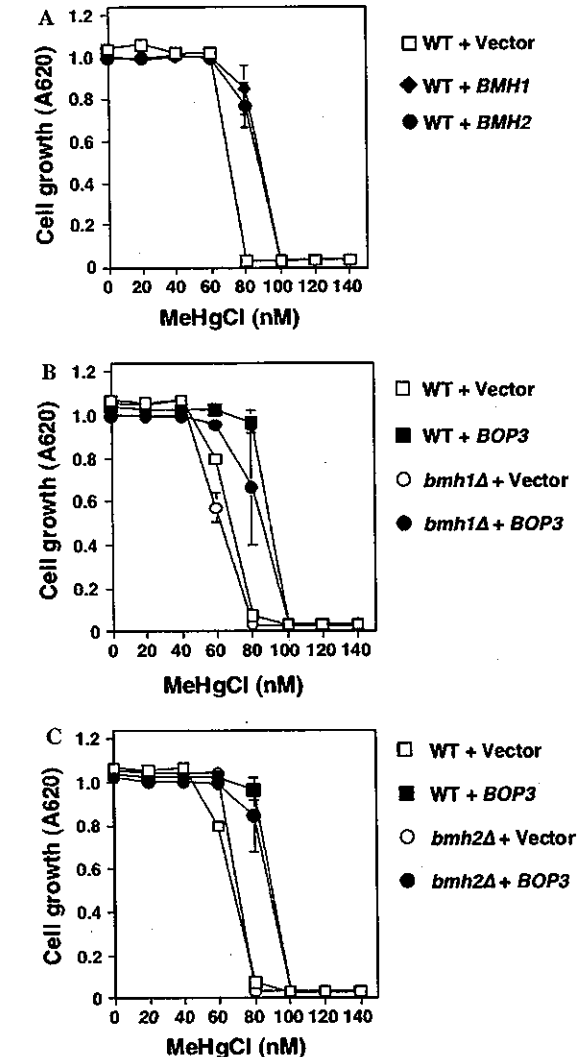


Fig. 3. Sensitivity of yeast cells overexpressing Bmh1 or Bmh2 to methylmercury (A), and effect of disruption of *BMH1* or *BMH2* gene on sensitivity to methylmercury of yeast that overexpressed Bop3 (B,C). (A) Yeast cells that harbored pKT10 (control), pKT10-BMH1 or pKT10-BMH2 were grown in SD (-Ura) liquid medium that contained methylmercuric chloride. (B,C) Yeast *bmh1Δ* (B) or *bmh2Δ* (C) cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (-Leu) liquid medium that contained methylmercuric chloride. For other details, see legend to Fig. 1B.

expressing Bmh1 or Bmh2—indeed, there were even clones that did not show resistance. It was reported previously that the growth of yeast overexpressing Bmh1 or Bmh2 was slow [18]. Thus, the level of Bmh mRNA in each clone was compared using quantitative PCR. It was found that the level of Bmh mRNA in the clones showing resistance to methylmercury was three to four times higher than in the controls. However, the mRNA level in clones not showing resistance to methylmercury was seven to eight times higher and the growth of the clones was found to be drastically reduced (data not

shown). On the basis of these findings, clones in which the *Bmh1* or *Bmh2* expression levels were three to four times higher than that of controls were used as yeast that overexpressed *Bmh1* or *Bmh2* for further study.

The effect of the deletion of *BMH1* or *BMH2* on the sensitivity of yeast to methylmercury was also examined. Virtually no change was observed in the sensitivity of the yeast to methylmercury when either *BMH1* or *BMH2* was disrupted (Fig. 3B). Upon overexpression of *Bop3*, the sensitivity of yeast deleted in either *BMH1* or *BMH2* to methylmercury was nearly the same as that of the wild-type strain after overexpression of *Bop3* (Fig. 3B). These results seem to indicate that *Bmh1* and *Bmh2* are not directly related to the methylmercury resistance conferred by overexpression of *Bop3*. However, because *Bmh1* and *Bmh2* share high homology (93%) with each other, and the yeast with disruptions in both *Bmh1* and *Bmh2* cannot grow [19], the possibility that the functional defects induced by deletion of either one of these proteins may be complemented by the other cannot be ruled out.

In addition to *Bop3*, several other proteins, such as *Msn2* and *Msn4* [28], are also known to bind to *Bmh1* and *Bmh2*. Because *Msn2* and *Msn4* are the transcription factors that respond to oxidative stress, changes in osmotic pressure, heat shock or malnutrition [29,30], it is conceivable that these proteins may exert some influence on the sensitivity of the cell to methylmercury. Therefore, the effect of overexpression of *Msn2* or *Msn4* on methylmercury sensitivity of yeast was examined. While sensitivity to methylmercury of yeast overexpressing *Msn4* was similar to that of the control, yeast that overexpressed *Msn2* showed a very high sensitivity to methylmercury (Fig. 4A). In contrast, although strains lacking *Msn2* (*msn2Δ*) showed some resistance to methylmercury, the degree of resistance was quite low (Fig. 4B). These results indicate that *Msn2* is not an essential factor for the development of resistance to methylmercury, although it is capable of augmenting the toxicity of this compound. To examine the involvement of *Msn2* in the acquisition of resistance to methylmercury by overexpression of *Bop3*, *Bop3* was overexpressed in the *msn2Δ* strain. The degree of resistance conferred by overexpression of *Bop3* in the *msn2Δ* strain was relatively low compared with that of the wild-type yeast (Fig. 4B). This result suggests the partial involvement of *Msn2* in the mechanism of resistance to methylmercury by overexpression of *Bop3*. *Msn2* is a transcription factor of the Cys2His2 Zn-finger type that localizes from the cytosol to the nucleus in response to various types of stresses. In the nucleus, *Msn2* activates transcription by binding to the stress response element (STRE) in the promoter region of the target gene [31]. High sensitivity to methylmercury brought about by overexpression of *Msn2* may result from an induction of one or more of these target genes. If this were

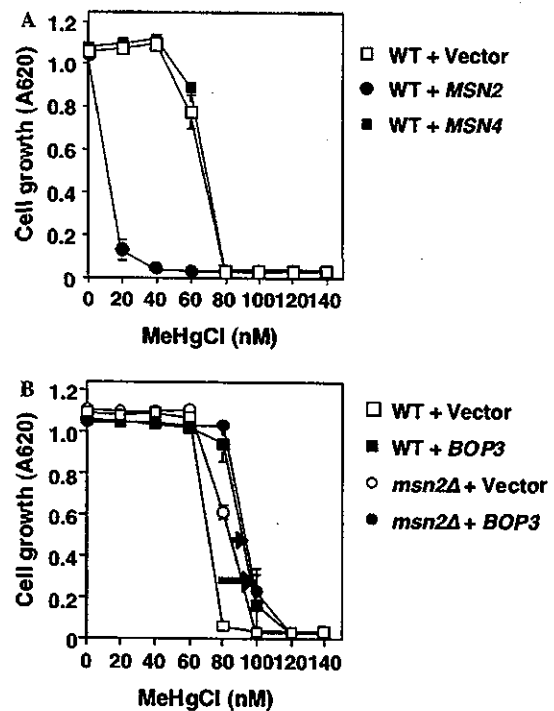


Fig. 4. Sensitivity of yeast cells that overexpressed *Msn2* or *Msn4* to methylmercury (A), and effect of disruption of *MSN2* gene on sensitivity to methylmercury of yeast that overexpressed *Bop3* (B). (A) Yeast cells that harbored pKT10 (control), pKT10-*MSN2* or pKT10-*MSN4* were grown in SD (-Ura) liquid medium that contained methylmercuric chloride. (B) Yeast *msn2Δ* cells, harboring pRS425 (control) or pRS425-*BOP3*, were grown in SD (-Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of *Bop3* in control yeast or in *msn2Δ* yeast. For other details, see legend to Fig. 1B.

the case, inhibition of either *Msn2* or its target protein(s) by *Bop3* might contribute to the acquisition of resistance to methylmercury by overexpression of *Bop3*.

Recently, *Fkh1* and *Rts1* were identified as binding proteins of *Bop3* by the two-hybrid method [14]. Forkhead homologue 1 (*Fkh1*) belongs to a family of transcription factors that have a DNA-binding domain of a winged-helix type called forkhead. It is known that transcription factors of the forkhead type participate in various intracellular responses such as early embryogenesis, differentiation, and the cell cycle [20]. Because the mRNA level of *G₂/M* cyclin is elevated in the *Fkh1*-deleted strain, participation of *Fkh1* in transcription silencing and pseudohyphal growth through cell cycle regulation is suggested [32]. *Rts1* is known to represent one of the B-subunits of serine-threonine phosphatase 2A (PP2A) [21–23]. PP2A is well conserved from yeast to human, where it is involved in a variety of intracellular responses such as DNA replication, transcription, signal transduction, and intermediary metabolism [33].

When Fkh1 or Rts1 was overexpressed in wild-type yeast, sensitivity to methylmercury was almost the same as that of the control (data not shown). While the methylmercury sensitivity of the Rts1-disrupted strain (*rts1Δ*) was nearly the same as that of the control strain (Fig. 5), the Fkh1-disrupted strain (*fkh1Δ*) did show resistance to methylmercury (Fig. 6). When Bop3 was overexpressed in *fkh1Δ*, the increased level of resistance to methylmercury was lower than in the wild-type strain overexpressing Bop3 (Fig. 6). However, overexpression of Bop3 in *rts1Δ* markedly increased the level of resistance to methylmercury in comparison to the wild-type strain (Fig. 5). Fkh1, combined with other factors, may increase the toxic effects of methylmercury. However, Fkh1 might

not be the rate-limiting factor in this scheme because overexpression of Fkh1 had little effect on the sensitivity of yeast to methylmercury. Fkh1 activity in the system might already be high in wild-type yeast, thereby nullifying the effect of its overexpression. Inhibition of Fkh1 activity by Bop3 may also be involved in the acquisition of resistance to methylmercury by overexpression of Bop3. In contrast, when Bop3 was overexpressed in *rts1Δ*, resistance to methylmercury was found to be greater than that observed when Bop3 was overexpressed in wild-type yeast (Fig. 5). This is despite the fact that deletion or overexpression of Rts1 did not affect the sensitivity of the yeast to methylmercury. Although the precise mechanism of action of Rts1 remains unknown, our results suggest that this protein suppresses the protective effect of Bop3 against methylmercury. It is conceivable that protein(s) with functions similar to those of Rts1 are present in the cell, and that the protein(s) exert inhibitory actions against the protective effects of Bop3 to methylmercury using the same mechanism. As the amount of the protein(s) present in wild-type yeast exceeded that necessary for inhibition of Bop3 activity, deletion or overexpression of Rts1 had no effect on the sensitivity to methylmercury. If this were the case, when the cellular amount of Bop3 exceeds the inhibitory activity of both Rts1 and the protein(s) with similar functions to Rts1, the excess Bop3 will elicit a protective effect against methylmercury. Furthermore, when Rts1 is deleted in Bop3-overexpressing cells, the resistance level of the cell to methylmercury is enhanced because the amount of Bop3 that evades the otherwise inhibitory activity of Rts1 is increased.

In the present study, overexpression of Bop3 was found to confer resistance to methylmercury in yeast. In addition, it was also found that proteins related to Bop3 (i.e., Bmh1, Bmh2, Msn2, Fkh1, and Rts1) participated in the augmentation or alleviation of methylmercury toxicity. Although Fkh1 and Rts1 are the proteins found to bind to Bop3 by screening with a two-hybrid method, there has been no report indicating the functional connection of these proteins to Bop3. The results of the present study show that Fkh1 and Rts1, as well as Msn2 (a Bmhs-binding protein), are involved in methylmercury toxicity. Disruption of the genes encoding each of these proteins influences the degree of acquisition of resistance to methylmercury in yeast overexpressing Bop3. This clearly demonstrates the involvement of these three proteins (Fkh1, Rts1, and Msn2) in the resistance mechanism to methylmercury brought about by overexpression of Bop3. It is conceivable that multiple systems are involved in the protective mechanism brought about by overexpression of Bop3 because deletion of Fkh1 or Msn2 only partially inhibits the protective effect of Bop3 against methylmercury. We believe that Bop3 participates in the regulation of an important intracellular function that exerts a protective effect

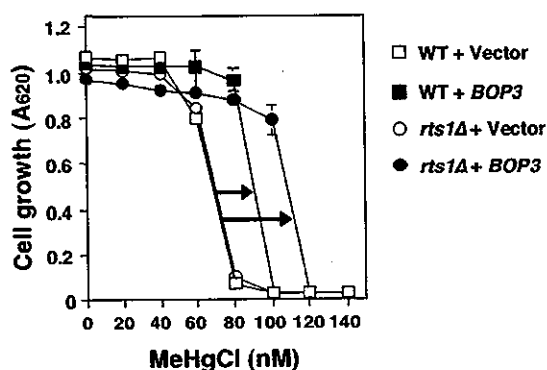


Fig. 5. Effect of disruption of *RTS1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *rts1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (-Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of Bop3 in control yeast or in *rts1Δ* yeast. For other details, see legend to Fig. 1B.

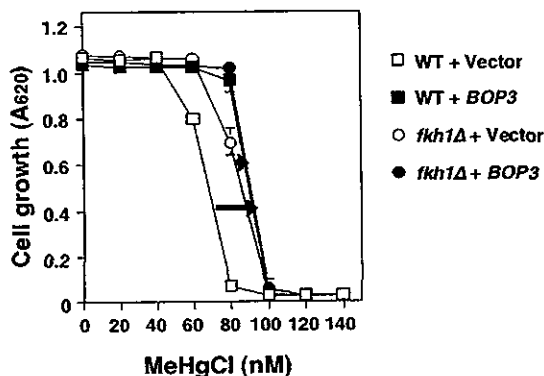


Fig. 6. Effect of disrupting the *FKH1* gene on sensitivity to methylmercury of control yeast or Bop3-overexpressing yeast. Yeast *fkh1Δ* cells, harboring pRS425 (control) or pRS425-BOP3, were grown in SD (-Leu) liquid medium that contained methylmercuric chloride. Arrows in the figure represent the magnitude of acquired resistance by overexpression of Bop3 in control yeast or in *fkh1Δ* yeast. For other details, see legend to Fig. 1B.

against methylmercury toxicity. It is anticipated that the protective effect of Bop3 against methylmercury, as reported for the first time in the present study, will contribute greatly to the elucidation of functions of this interesting protein.

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