

Overexpression of L-glutamine:D-fructose-6-phosphate amidotransferase provides resistance to methylmercury in *Saccharomyces cerevisiae*

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Abstract To identify novel genes that confer resistance to methylmercury (MeHg), a yeast genomic DNA library was transfected into *Saccharomyces cerevisiae*. Two functional plasmids were isolated from transfected yeast clones D1 and H5 that exhibited resistance to MeHg. The yeast transfected with plasmid isolated from clone H5 was several-fold more resistant than yeast transfected with plasmid from clone D1. Functional characterization of the genomic DNA fragment obtained from clone H5 determined that the *GFAI* gene conferred resistance to MeHg. *GFAI* was reported to encode L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) which catalyzes the synthesis of glucosamine-6-phosphate from glutamine and fructose-6-phosphate. Accumulation of mercury in yeast clone W303B/pGFA1, which contains the transfected *GFAI* gene, did not differ from that in control yeast clone W303B/pYES2. The W303B/pGFA1 strain did not show resistance to mercuric chloride, zinc chloride, cadmium chloride or copper chloride, suggesting that the resistance acquired by *GFAI* gene transfection might be specific to MeHg. This is the first report of a gene involved in MeHg resistance in eukaryotic cells identified by screening a DNA library.

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1. Introduction

Methylmercury (MeHg) is one of the most significant environmental pollutants and causes neurological toxicity that affects the function of areas such as the cerebellum and the visual cortex [1,2]. The cytotoxicity of MeHg may be modified by some cellular factors. The degree of expression of the MeHg toxicity depends on the type of cells [3]. Cell lines resistant to MeHg can be obtained by keeping cells in MeHg containing medium [4,5]. Glutathione has been considered as one of the factors affecting the expression of MeHg toxicity [4]. Overexpression of manganese-superoxide dismutase (Mn-SOD) has also been reported to give HeLa cells resistance to MeHg toxicity [6]. However, these factors were identified by studies on some known substances or enzymes responsible for protection of cells against the toxicity of

foreign substances. In the present study, we attempted to search for novel genes conferring MeHg resistance by transfecting a yeast genomic DNA library into *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Transfection of yeast with a genomic DNA library

The genomic DNA of *S. cerevisiae* was partially digested with *Sau3AI* and inserted into the *BamHI* site of LEU2-based multicopy plasmid YEp13 to obtain a yeast genomic DNA library (a kind gift from Dr Paul Russel). Yeast was transformed by the lithium acetate method. Leucine auxotrophic yeast W303B (*MAT α leu2 his3 can1-100 ade2 ura3*) was seeded in 50 ml of yeast-peptone-adenine-dextrose medium (1% bacto-yeast extract, 2% bacto-pentone, 2% glucose, 40 mg/l adenine) and was cultured with shaking until the cell density reached 2×10^7 cells/ml. The cells were collected and suspended in 1 ml of 100 mM lithium acetate solution. To this solution, 1 μ g of the yeast genomic DNA library, 50 μ g of heat-denatured salmon sperm DNA and 300 μ l of 50% polyethyleneglycol 4000 were added and the resulting solution was incubated for 30 min at 30°C. After incubation, the cells were heat-shocked for 15 min at 42°C and then collected. The cells were seeded on a leucine-free synthetic minimal medium (SDM (–leu), 0.67% yeast nitrogen base, 2% glucose, 40 mg/l adenine, 20 mg/l histidine, 60 mg/l leucine, 40 mg/l tryptophan, 20 mg/l uracil, 1.3 g/l dropout powder) and cultured at 30°C for 3 days.

2.2. Screening of yeast cells transfected with a MeHg resistance gene

The obtained Leu⁺ transformants were seeded at a cell density of 5×10^3 cells per plate on SDM (–leu) agar plates containing MeHg chloride at a concentration (20 nM) that inhibited the growth of W303B. After incubation for 3 days at 30°C, grown colonies were collected.

2.3. Recovery of plasmid DNA from yeast cells and amplification in *Escherichia coli*

Plasmid DNA was recovered from the yeast using glass beads. First, single colonies were seeded in 2 ml of SDM (–leu) medium and incubated for 24 h at 30°C. Then, cells were collected and suspended in 200 μ l of breaking buffer (pH 8.0, 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA). To this solution, 200 μ l of phenol/chloroform/iso-amylalcohol (25:24:1) and 0.3 g of acid-washed glass beads were added. The solution was vigorously stirred for 5 min. Following centrifugation at $12\,000 \times g$ for 5 min, 200 μ l of aqueous phase containing plasmid DNA was obtained. The obtained DNA solution was concentrated by ethanol precipitation to a final volume of 10 μ l. *E. coli* (XL-1 blue strain) was transfected with plasmid DNA by the method reported by Hanahan et al. [7]. To 200 μ l of competent cell suspension, 5 μ l of the above DNA solution was added. After the cells had stood on ice for 30 min, they were heat-shocked at 42°C for 30 s and kept standing on ice for 1 min. To this solution, 0.8 ml of SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl, 20 mM MgCl₂, 20 mM glucose) was added and then, the cells were incubated for 1 h at 37°C. This culture solution was smeared on LB medium (pH 7.5, 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) containing 50 μ g/ml of ampicillin and cultured at 37°C overnight. The grown colonies were cultured further in LB medium containing 50 μ g/ml

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ampicillin with shaking overnight and plasmid DNA was recovered from the *E. coli* using the Wizard Minipreps DNA Purification System (Promega, Madison, WI, USA).

2.4. Sensitivity of yeast re-transfected with plasmid to MeHg

Single colonies of the yeast re-transfected with the plasmid were seeded in 2 ml of SDM (–leu) medium and cultured at 30°C overnight. The culture fluid was diluted with SDM (–leu) medium to adjust the cell density to 5×10^6 cells/ml and cultured for 5 h at 30°C. At the mid-logarithmic growth phase, the secondary transfectants were seeded at a density of 5×10^6 cells per plate on SDM (–leu) agar medium containing MeHg chloride and examined for colony formation after 3 days of culturing at 30°C. Yeast transfected with the vector YEp13 was used as a control.

2.5. Restriction enzyme mapping

Plasmid isolated from clone H5 was digested with several appropriate restriction enzymes. The cleavage patterns were analyzed by electrophoresis on a 0.6 or 2% agarose gel and a restriction enzyme map of the yeast genomic DNA fragment insert was prepared.

2.6. Subcloning in yeast expression vector

The plasmid isolated from clone H5 was digested with *Bam*HI and *Hind*III and the resulting fragment was separated by 0.8% agarose gel electrophoresis. The fragment was cut out of the agarose gel and purified using the Gene Clean III kit (Bio 101, Vista, CA, USA). The yeast expression vector pYES2 was also digested with *Bam*HI and *Hind*III. Then, the fragment was ligated with pYES2 using the DNA Ligation kit ver.2 (Takara, Otsu, Japan) followed by amplification in *E. coli*, to create plasmid pYES2 with an inserted *Bam*HI or *Hind*III fragment.

2.7. Partial nucleotide sequence determination

The nucleotide sequences were determined for both ends of the *Bam*HI fragment by the dideoxy cycle sequencing method. First, the *Bam*HI fragment was subcloned in pBluescript II (Stratagene, La Jolla, CA, USA) and used as a template DNA. To this template DNA (400 ng), 3.2 pmol of primer for sequencing (M13 Primer M3, 5'-GTAAACGACGGCCAGT-3' or M13 Primer RV, 5'-CAG-GAAACAGCTATGAC-3', Takara) and 8 µl of Terminator Ready Reaction Mix (Perkin Elmer) were added. The solution was adjusted to a final volume of 20 µl with sterile distilled water and the fragment was amplified by PCR. After the PCR product was concentrated by ethanol precipitation, the product was dissolved in 4 µl loading buffer (25 mM EDTA, 50 mg/ml blue dextran/formamide 1:6) and heat-treated at 90°C for 2 min followed by rapid cooling in an ice bath. This product was separated on a 6% sequencing gel (Pageset SQC-6A,

Biomate) and the nucleotide sequences were analyzed using an auto-sequencer (ABI 373A, Perkin Elmer).

2.8. L-Glutamine:D-fructose-6-phosphate amidotransferase (GFAT) activity measurement

To 50 µl of the yeast extract, 450 µl reaction solution (6 mM D-fructose-6-phosphate, 12 mM L-glutamine, 1.25 mM EDTA, 40 mM sodium phosphate buffer (pH 7.5)) was added and incubated at 37°C for 2 h. Then, the solution was heated in boiling water for 3 min to stop the enzyme reaction, centrifuged at $12\,000 \times g$ for 10 min and the supernatant (0.4 ml) was obtained. Glucosamine-6-phosphate in the supernatant was measured by the modified Elson-Morgan method [8].

3. Results and discussion

The gene that confers MeHg resistance to yeast was sought in a yeast genomic DNA library. The yeast genomic library using 2 µm system multicopy plasmid YEp13 as the vector was introduced into yeast cells (W303B) by the lithium acetate method. Approximately 2×10^4 transformants were found to highly express the transfected gene. These transformants were seeded on agar medium containing MeHg at a concentration of 20 nM and grown for 3 days. Growth of the parent strain was almost completely inhibited, but 10 transfectants (D1–D5 and H1–H5) were able to grow under such conditions. Plasmids were recovered from these clones and re-transfected into the parent strain. Most yeast cells transfected with plasmids from clones D1 and H5 were resistant to MeHg. The yeast transfected with plasmid from H5 was several-fold more resistant to MeHg than those transfected with plasmid from D1 (Fig. 1). The MeHg resistance of clones other than D1 and H5 may have been due to mutation or transient acquisition of resistance, rather than gene transfection.

To investigate the size and restriction enzyme sites of the yeast genomic DNA fragment inserted in the plasmid from H5 clone, which exhibited the highest MeHg resistance, the fragment was digested with several appropriate restriction enzymes and analyzed by agarose gel electrophoresis. The length of the yeast genomic DNA fragment inserted into the plasmid from clone H5 was approximately 9.2 kb in length and contained two *Bam*HI sites and one *Hind*III site (Fig. 2A). Since

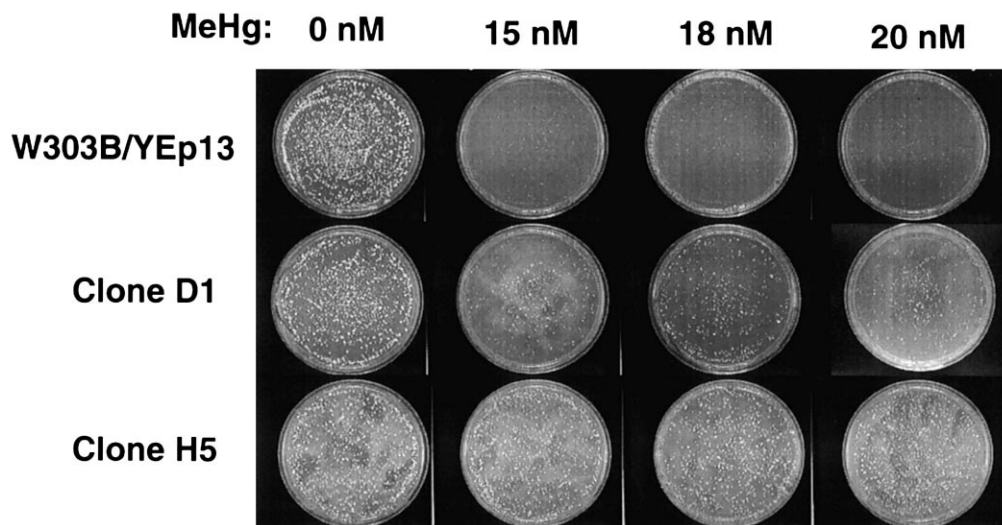


Fig. 1. Sensitivity to MeHg of yeast re-transfected with plasmid from MeHg resistant clone D1 or H5. Clones D1 and H5 were selected as MeHg resistant clones after transfection of the genomic DNA library (YEp13 was used as a vector) into the W303B yeast strain. Yeast transfected with the vector YEp13 was used as control.

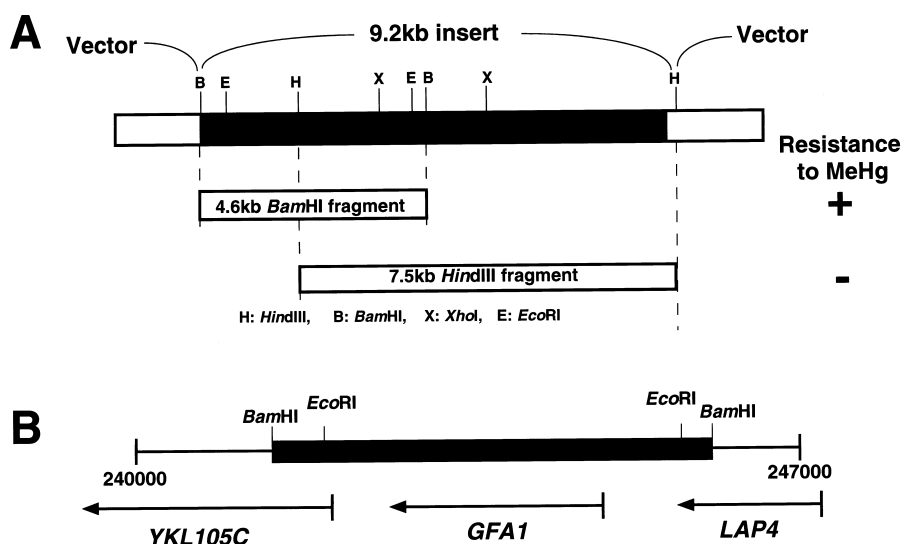


Fig. 2. Restriction map of a genomic DNA fragment inserted in the plasmid from clone H5 (A) and location of ORFs in its *Bam*HI fragment (B).

the long size of the insert, 9.2 kb, implied the presence of multiple genes, the insert region was cleaved into a *Bam*HI fragment of approximately 4.6 kb and a *Hind*III fragment of approximately 7.5 kb, to narrow the region involved in MeHg resistance. After purifying the *Bam*HI and *Hind*III fragments from the agarose gel, these two fragments were subcloned in multicopy plasmid pYES2. The parent strain was transfected with these vectors containing the *Bam*HI and *Hind*III fragments and examined for MeHg resistance. The yeast transfected with *Hind*III fragment did not form colonies in the presence of 15 nM MeHg. In contrast, yeasts transfected with the *Bam*HI fragment formed colonies in the presence of 20 nM MeHg, showing clear resistance. Therefore, within the 9.2 kb insert, the region involved in MeHg resistance was present in this *Bam*HI fragment.

Since the entire genomic DNA sequence of *S. cerevisiae* is known, nucleotide sequences approximately 300 bp in length were determined at both ends of the H5 *Bam*HI fragment and matches were searched for in the *Saccharomyces* Genome Database. The H5 *Bam*HI fragment obtained in this study corresponded to nucleotide sequences 241 442–246 087 of yeast chromosome XI (Fig. 2B). This region contains three open reading frames (ORFs) of *YKL105C*, *GFA1* and *LAP4*. Only *GFA1* was completely contained within the H5 *Bam*HI

fragment, suggesting that it is the gene involved in MeHg resistance. Therefore, we isolated *GFA1* as a 3.7 kb *Eco*RI fragment and subcloned it in pYES2 and then transfected it into yeast (W303B). Examination of the MeHg sensitivity of the transfectants demonstrated that the yeast transfected with the *Eco*RI fragment was clearly as resistant as yeast transfected with the *Bam*HI fragment described above. Based on these findings, *GFA1* may be one of the yeast genes involved in MeHg resistance. *GFA1* has been reported to encode GFAT (EC 2.6.1.16) [9]. GFAT catalyzes the synthesis of glucosamine-6-phosphate from glutamine and fructose-6-phosphate and is conserved in a wide range of living organisms from microorganisms to mammals, such as *E. coli*, mice, rats, humans and yeasts [8,10]. Since glucosamine-6-phosphate produced by a GFAT-catalyzed reaction is the starting material of all amino sugar biosynthesis pathways, GFAT is considered as an essential enzyme for the growth and functional maintenance of cells. In this study, yeast transfected with the *GFA1* gene (W303B/pGFA1) showed an approximately 5-fold higher GFAT activity than those in the parent strain and the yeast transfected with vector alone (W303B/pYES2) (Fig. 3), suggesting that elevation of GFAT activity results in MeHg resistance in the yeast.

The W303B/pGFA1 strain was incubated with MeHg for

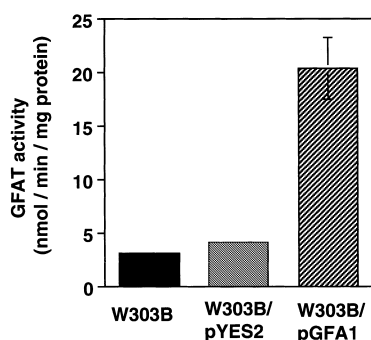


Fig. 3. GFAT activity of yeast transfected with the *GFA1* gene. The *GFA1* gene was inserted in pYES2 vector and transfected into the W303B yeast strain.

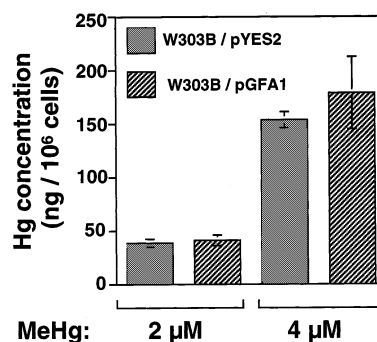


Fig. 4. Accumulation of mercury in yeast transfected with the *GFA1* gene. The mercury concentration was determined after 4 h incubation with MeHg.

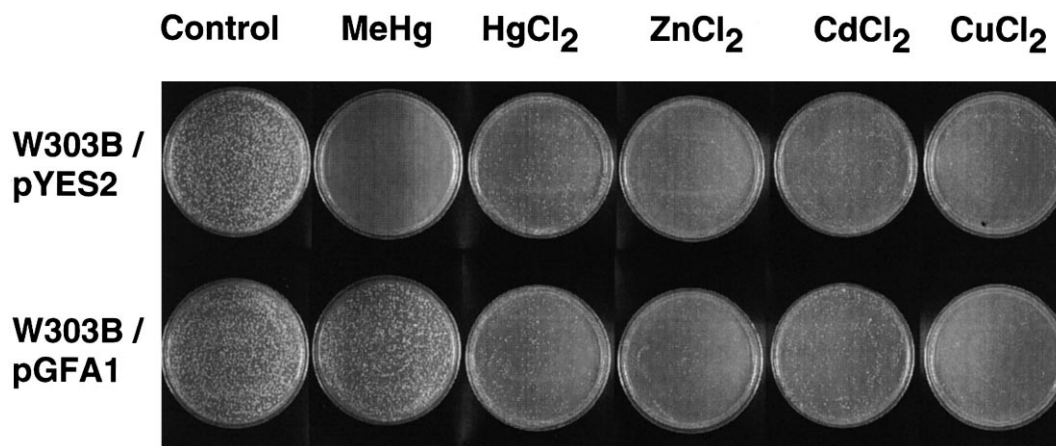


Fig. 5. Sensitivity of yeast transfected with the *GFA1* gene to heavy metal compounds. W303B/pYES2 and W303B/pGFA1 were treated with the minimum lethal concentration of MeHgCl (20 nM), HgCl₂ (400 nM), ZnCl₂ (10 mM), CdCl₂ (30 μM) or CuCl₂ (2 mM) for 3 days.

4 h and the intracellular mercury concentration was measured using a mercury analyzer. There were no significant differences in the mercury concentration between the W303B/pGFA1 and parent yeast (Fig. 4). This finding indicates that high expression of *GFA1* does not affect the incorporation or excretion of MeHg. The W303B/pGFA1 strain did not show resistance to the minimum lethal concentration of mercuric chloride, zinc chloride, cadmium chloride or copper chloride (Fig. 5), suggesting that the resistance acquired by *GFA1* gene transfection may be specific to MeHg. GFAT is an SH enzyme and its activity is known to be inhibited by mercury compounds [11]. In the present study, inhibition of GFAT activity of wild-type yeast (W303B) by treatment with MeHg was observed in a dose-dependent manner (data not shown). There is a possibility that GFAT is an intracellular target of MeHg. Thus, overexpression of GFA1, the gene encoding GFAT, may lead to the increased resistance to MeHg seen in the present study.

Although Mn-SOD has been observed as a resistance factor for MeHg toxicity among some antioxidant genes [6], this is the first report, to our knowledge, that identified a cellular

factor involved in MeHg resistance in eukaryotic cells by screening a DNA library.

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