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Cloning of the Genomic DNA Which Complements the Drug-Hypersensitivity of *Saccharomyces cerevisiae*

Yun-Sik Lee and Kie-In Park[1]*

National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305, Japan
[1]Department of Biology, College of Natural Science, Chonbuk National University, Chonju 560-756, Korea
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Abstract : The yeast *Saccharomyces cerevisiae*, mutant CH117, shows a drug-hypersensitivity (*dhs*) to cycloheximide, bleomycin, actinomycin D, 5-fluorouracil, nystatin, nigericin and several other antibiotics. CH 117 was also temperature-sensitive (*ts*), being unable to grow at 37°C and secreted more invertase and acid phosphatase into the medium than the parent yeast. CH117 grows very slowly and the cell shape is somewhat larger and more sensitive to zymolyase than the wild type cells. Light microscopic and electron microscopic observation also revealed abnormality of the mutant cell wall. These characteristics indicate that CH117 has a defect in an essential component of the cell surface and that the cell wall which performs barrier functions has become leaky in the mutant. We screened a genomic library of wild type yeast for clones that can complement the mutation of CH117. A plasmid, pCHX1, with an insert of 3.6 kilobases (kbs) could complement the *dhs* and *ts* of CH117. Deletion and subcloning of the 3.6 kb insert showed that a gene for the complementation of mutant phenotypes was located in 1.9 kbs *PvuII-HindIII* fragment.
Key words : drug-hypersensitivity, *Sacchromyces*, temperature-sensitive

The yeast cell envelope has been postulated to contain at least three classes of mannoprotein: the inducible hydrolytic enzymes, such as external invertase and acid phosphatase, the sexual agglutinins, expressed on cells that are homozygous at the mating-type locus, and, lacking a better term, structural mannoproteins (Ballou, 1976; Kollar *et al.*, 1995). The chemical structure of cell wall of *S. cerevisiae* consists of three main kinds of polymer (Cabib *et al.*, 1982): glucan (mainly of the β-glucan type), which forms the structural network of the wall; chitin, a minor structural polymer present on the bud scars and near the plasma membrane in smaller amounts (Horisberger *et al.*, 1977); and glycoprotein molecules usually formed by a mannose polysaccharide (mannan) covalently linked to protein moieties. While glucan supports and maintains the rigidity of the cell wall, the mannoprotein determines its permeability (Zlotnik *et al.*, 1984). The chemical structure of wall mannan in *S. cerevisiae* and some other yeast species is well known. Microfibrils of β-(1,3)-glucan make up the wall fibrillar skeleton, which is masked with amorphous β-(1,3)-glucans and mannoproteins (Pastor *et al.*, 1984; Kopecka, 1985; Hartland *et al.*, 1993). In vitro enzymatic reactions resulting in glucan synthesis have been defined and partially characterized for several systems (Kang and Cabib, 1986), although components of the synthetic machinery have eluded purification (Ram *et al.*, 1994). Glucanases do not work effectively unless mannoproteins are degraded by protease(s) present in the lytic enzymes such as Zymolyase (Zlotnik *et al.*, 1984). Secretory enzymes such as invertase or acid phosphatase are not excreted in the medium unless the wall is removed by lytic enzyme. Thus the cell wall of *S. cerevisiae* has a barrier function (Scherrer *et al.*, 1974). Chitin is almost exclusively present in bud scars (Bacon *et al.*, 1966). Although extensive studies have been done on the cell surface, the precise structure and functions remain far from complete understanding. The isolation of mutants defective in the production of cell wall components should define genes that encode biosynthetic enzymes as well as other products, for exemple that regulate glucan synthesis or generate glucan precursors (Boone *et al.*, 1990). A mutant approach has been valuable in understanding the synthesis of such other cell wall polysaccharide, as glucan, mannan and chitin (Shiota *et al.*, 1985; Bulawa *et al.*,

*To whom correspondence should be addressed.
Tel : 82-652-70-3361, Fax : 82-652-70-3362
E-mail : park253@moak,chonbuk.ac.kr

1986; Ram *et al.*, 1994; Shimizu *et al.*, 1994).

In this paper, we report the isolation and characterization of a mutant of *S. cerevisiae* which has a defect in the barrier function of the cell surface and cloning of the responsible yeast genomic DNA, *CHX1* (Cycloheximide sensitive) gene, which complements the *dhs* and *ts* of the cell wall defective mutant, CH117.

Materials and Methods

Strains

Escherichia coli K12 strains, MC1061 (F^-, *aro*D139, *lac*X74, *gal*U, *gal*K, *hsd*M, *hsd*R, *str*A) and JM109 (*recA 1, lac-pro, endA1, gyrA1, thi, hsdR17, supE44, relA1, F' (traD36, proA⁺B⁺, lacIZM15)*) were used for all bacterial transformation and plasmid propagations. The yeast *Saccharomyces cerevisiae* strain YSL37 (*MATa, trp1, ura3*) was constructed by a cross between A5-8-1C (*MATα, leu1*) and HN18 (*MATα, leu2, trp1, ura3, suc2*). The cell wall defective mutant, CH117 was obtained from YSL37 by MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) mutagenesis.

Plasmids

E. coli plasmids, pUC118, pUC119 and an *E. coli-S. cerevisiae* shuttle vector pYES2.0 (Invitrogen, San Diego, USA) were used. A yeast genomic library was a kind gift from Y. Ohya (Department of Botany, University of Tokyo). This library was constructed by inserting *Sau*3A 1 fragments of A5-8-1A (*MATa, leu1*) chromosomal DNA in the *Bam*HI site of a centromere vector YCpG11 (Ohya *et al.*, 1986).

Plasmid DNA was isolated by alkali-SDS method (Sambrook *et al.*, 1989). Transformation was done by $CaCl_2$ method in *E. coli* (Sambrook *et al.*, 1989) and Li-acetate method in *S. cerevisiae* (Ito *et al.*, 1983; Becker and Guarente, 1991).

Chemicals and enzymes

MNNG, antibiot- ics, and invertase were purchased from Sigma Chemical Co. (St. Louis, USA). Restriction enzymes, T4 DNA ligase, DNA polymerase I Klenow fragment, exonuclease III and mung bean nuclease were purchased from Takara Shuzo Co. (Kyoto, Japan) or Boehringer Mannheim Co. (Mannheim, Germany). Yeast cell wall lytic enzyme, Zymolyase 100T, was obtained from Seikagaku Kogyo Co. (Tokyo, Japan).

Media

Bacteria were grown in nutrient broth (18 g/l, Eiken, Tokyo, Japan). MY medium contained 10 g glucose, 3 g malt extract, 3 g yeast extract, 5 g peptone, and 50 mg uracil per liter. YEPD medium contained 20 g glucose, 20 g peptone, 10 g yeast extract and 50 mg uracil per liter, YEPS medium containined 4 g glucose, 20 g sucrose, 20 g peptone, 10 g yeast extract and 50 mg uracil per liter. Minimal (YNB) medium contained 0.67 g yeast nitrogen base without amino acids (Difco), 20 g glucose per liter and was supplemented with 50 mg/l uracil or tryptophan if necessary. Sporulation medium contained 10 g potassium acetate, 1 g yeast extract and 0.1 g glucose per liter. Media were solidifed with 20 g/l of agar (Kokusan Kagaku Co., Tokyo, Japan).

Mutagenesis

Mutagenesis was performed as described (Lewrence, 1991) with a slight modification. Five ml each of logarithmic phase cultures of YSL 37 at a density of $3\text{-}5 \times 10^8$ cells/ml was spun down, washed twice with same volume of 0.1 M citrate buffer (pH 5.5), and resuspended in 4 ml of the same buffer. Three hundred μl of 10 mg/ml solution of MNNG in methanol was added and kept at 30℃ for 30 minutes. Cells were washed with 0.1 M phosphate buffer (pH 7.0) and grown in 10 ml YEPD overnight at 25℃. Mutagenized cells were collected and suspended in distilled water for longer storage at 4℃.

Production of enzyme

The invertase and acid phosphatase were assayed by the methods described elsewhere (Luchsinger and Cornesky, 1962; Nakada and Shinagawa, 1986).

Gel Electrophoresis and activity staining of invertase

Native gel electrophoresis and activity staining was performed on 5% polyacrylamide gel as described by Ballou (1990).

Electron microscopy

The conditions described below represent a modified version of the procedure published by Zlotnik *et al.* (1984). Cells were grown in YEPD media to stationary phase, harvested and washed with distilled water. Cell pellets were fixed in a solution containing 2% $KMnO_4$ for 90 min, rinsed with distilled water 5 times and then embedded in 2% Bacto-agar at 60℃. Samples were subsequently dehydrated through a grade ethanol and acetone series embedded in modified Spurr's epoxy resin and polymerized at 70℃ for 30 hours (Spurr, 1969; Kushida, 1981). Silver-gold-colored sections were mounted on formvar-coated grids, stained with uranyl acetate and lead citrate. Sections were viewed with a JEOL 200CX electron microscope at an operating voltage of 100 kV.

Molecular cloning

General methods were as described by Sambrook, *et*

al. (1989) and Guthrie and Fink (1991). The yeast genomic library constructed with the single copy shuttle vector YCpG11 was used for the screening for the gene complementing the mutant phenotype of CH117. Transformation was performed with Li-acetate method (Ito *et al.*, 1983; Becker and Guarente, 1991) and Trp^+ transformants were selected on YNB agar plates. Transformants were replica plated on YEPD agar plates containing 100 ng/ml of cycloheximide. From the candidate tranformants plasmid DNAs were extracted by mini-preparation and used for transformation of *E. coli* MC1061. After propagation, *E. coli* MC1061 cells containing this plasmid were detected by their ability for colony formation on NB plates containing 100 μg/ml ampicillin and used as the source of DNA for all subsequent complementary studies.

Results and Discussion

Isolation of mutants

Defective mutants of essential genes could only be obtained as conditional lethal mutants. However, temperature-sensitive mutants often show partial defects even at a permissive temperature.

We presumed that mutants with defects in the cell surface structure will become more sensitive to various antibiotics and excrete more periplasmic enzymes to the medium because barrier function of the cell wall will be damaged. Furthermore, if the mutated gene product is essential for yeast growth, we also might expect the mutants are lethal at higher temperatures. Thus we planned to isolate drug-hypersensitive and temperature-sensitive mutants of *S. cerevisiae*.

Among 7,200 mutagenized colonies grown on YEPD at 30℃, 461 colonies did not grow on YEPD containing 100 ng/ml of cycloheximide at 37℃ while the parent YSL 37 grew well under these conditions. Among them, 51 colonies did not grow on YEPD containing cycloheximide at 30℃, 54 colonies did not grow on YEPD without the antibiotics at 37℃, and 13 colonies did not grow under either conditions. We further examined for their sensitivities to other drugs and for excretion of periplasmic enzymes by replica plating as preliminary tests for the leakiness of the surface barrier function and selected one mutant, CH117, for further study.

CH117 showed no growth on YEPD containing 100 ng/ml of cycloheximide at 30℃ or YEPD without cycloheximide at 37℃. CH117 grows very slowly in YEPD even at 30℃ and was a non-growing at the non-permissive temperature (Fig. 1). The mutation was quite stable and revertants appeared at a frequency below 10^{-7}. As diploids constructed by mating CH117 with A 5-8-1C were neither *dhs* nor *ts*, the mutant alleles are recessive to wild type alleles. This allowed us to clone the wild type allele by transformation of a yeast genomic library.

Characteristics of mutant

We next examined further the sensitivity of CH117 to various antibiotics with various blocking points such

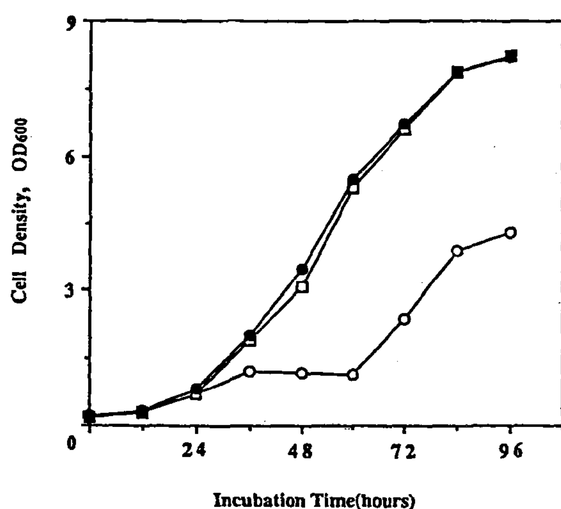

Fig. 1. Growth of the cell wall defective mutant, CH117. The mutant CH117, wild type yeast YSL37 and transformant with yeast genomic library CH117/pCHX1 were incubated in YEPD without cycloheximide at 30°C. Open circle, closed circle and square indicate mutant, wild type, and transformant, respectively.

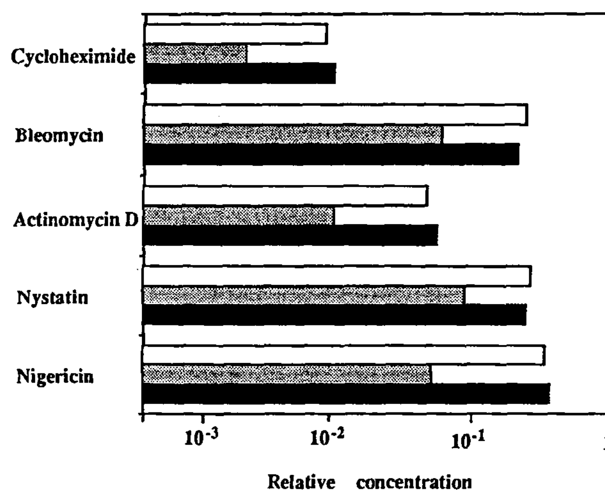

Fig. 2. Drug sensitivity of the mutant, CH117 and its complementation by pCHX5. Logarithmically growing cultures of *S. cerevisiae* YSL37, CH117, or CH117/pCHX5 were diluted in YEPD to about 1,000 cells/ml and used to make dilutions of drugs, 3-fold each step. The highest concentration of drug are 100 μg/ml for cycloheximide, 500 μg/ml for bleomycin, 100 μg/ml actinomycin D, 100 μg/ml for nystatin and 50 μg/ml for nigericin. Samples were incubated at 30°C and growth of the cells were examined. Open bar, dotted bar and closed bar indicate wild type, mutant, and transformant, respectively.

as DNA synthesis, RNA synthesis, protein synthesis or membrane functions. Drugs were serially diluted by 3-fold each in YEPD liquid culture containing about 1000 cells/ml of YSL37 (the parent), CH117 (the mutant) or CH117/pCHX1 (the mutant carrying the plasmid which complements the mutation, see below). We used 96-well microtitration plates and incubated at 30℃. The highest concentration of the antibiotics were 100 μg/ml for cycloheximide, 500 μg/ml for bleomycin, 100 μg/ml for actinomycin D, 100 μg/ml for nystatin and 50 μg/ml for nigericin. CH117 was more sensitive than the wild-type to all the antibiotics tested (Fig. 2). This indicates CH117 has a general leakiness in the barrier function because the lethal target such as DNA synthesis, RNA synthesis, protein synthesis or membrane function of these antibiotics are quite different. CH117 showed higher sensitivity to wall lytic enzyme than the YSL37 parent and the transformant. The transformant of CH117 with pCHX1 showed the same sensitivities as the parent.

Enzyme production

Preliminary assay on plates and polyacrylamide gel electrophoresis for the production of two periplasmic enzymes, invertase and acid phosphatase, indicated the mutants might excrete larger amount of the enzymes than the wild type. We assayed the amounts of the enzymes in the medium in liquid cultures by absorbances and polyacrylamide gel electrophoresis (Data not shown). As shown in Fig. 3 and Fig. 4, CH117 excreted larger amounts of the enzymes in the medium than YSL37 parent and CH117/pCHX5 transformant (see below) even though the cell density was lower than the others. These results also indicate the barrier function of the cell surface is affected by the mutation.

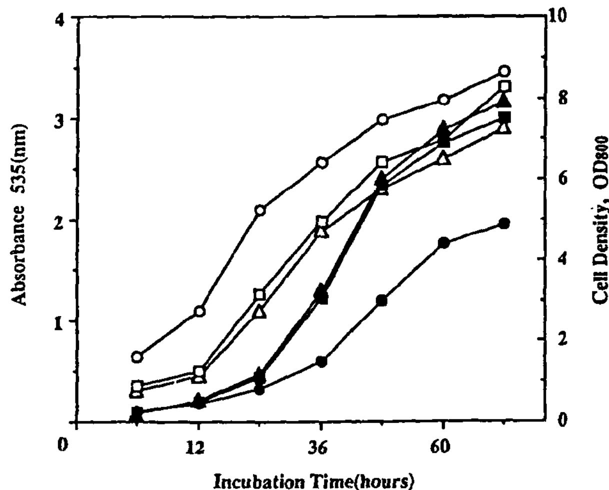

Fig. 3. Secretion of invertase from the mutant. To compare enzyme secretion of the mutant with that of wild type YSL37 and transformant CH117/pCHX5, the cells incubated in YEPS broth were spun down. The surpernant was assayed by using a dinitrosalicylic acid reducing power at A_{535}. Closed symbols indicate growth of cells. Open symbols indicate secretion of invertase from cells. Circle, triangle and square indicate mutant, wild type and transformant, respectively.

Morphology and fine structure of the mutant

Observation of the mutant and the wild type cells by a phase-contrast microscopy revealed a change in cell shape. Most of the mutant cells showed irregular shapes with swelling and wrinkling (data not shown). The abnormality of cell morphology was also complemented by pCHX5 indicating the same mutation affected the cell

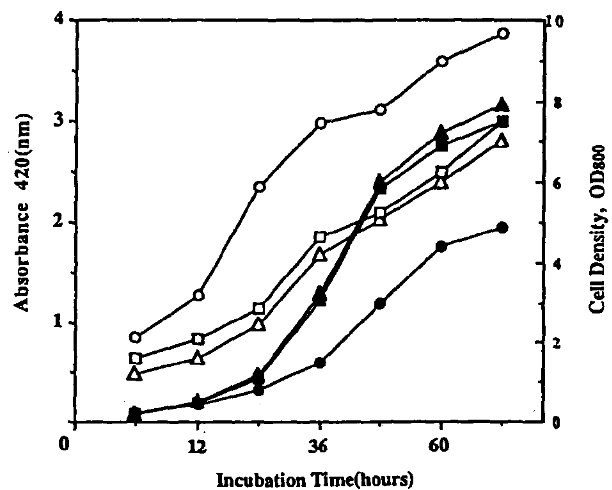

Fig. 4. Secretion of acid phosphatase from the mutant CH117. To compare acid phosphatase secretion of the mutant with wild type YSL37 and transformant CH117/pCHX5, the cells incubated in Burkholder broth were spun down. The surpernant was assayed by using a n-nitrophenyl phosphate(NPP) solution at A_{420}. Closed symbols indicate growth of cells. Open symbols indicate secretion of invertase from cells. Circle, triangle and symbols indicate mutant, wild type and transformant, respectively.

Fig. 5. Electron microscopic observance of cell shapes and wall structure. Wild type YSL37 (a) and the cell wall defective mutant CH117 (b). Bar is 0.2 μm.

morphology. But we could not find irregularity of nucleus by DAPI nuclear staining (photograph not shown).

Electron microscopic observation also revealed an abnormality of the cell shape and cell wall structure of CH117. The cell wall of CH117 was swelled and irregular in form and seemed to be less rigid than the wild type cell wall. The thickness of the mutant cell wall is estimated to be ca. 3 mm, twice as thick as the wild-type cell wall (Fig. 5). This abnormality was also complemented by pCHX1. The *dhs*, *ts* and excretion of periplasmic enzyme by the mutation of CH117 would be caused by this abnormality of the cell wall. Further biochemical studies should reveal the primary event in this abnormality.

Cloning of the complementing DNA

In an attempt to clarify the molecular aspects of the mutation, we screened a genomic library of wild type yeast that can complement the mutation of CH117. The yeast genomic library was constructed by inserting random *Sau*3A1 fragments of the chromosomal DNA of A5-8-1A in the *Bam*HI site of a single copy shuttle vector YCpG11 (Ohya *et al.*, 1986) We transformed CH117 with the library DNA by Li-acetate method and Trp+ transformants were selected on YNB agar plates. Fifty-six transformants were replica plated on YEPD agar plates containing 100 ng/ml of cycloheximide at 37℃. From the candiate transformants plasmid DNAs were extracted by mini-preparation and used for transformation of *E. coli* MC1061. After propagation in *E. coli*, the plasmid DNAs were used for transformation of CH117 to confirm the complementing activity. Thus, plasmid DNAs were recovered from colonies grown on this plate and checked for the complementing activity after propagation in *E. coli*. All plasmids that complemented the cycloheximide sensitivity of CH117 contained a common 3.6 kb insert. We named one of them pCHX1 and used it for further study.

CH117 did not grow on YEPD at 37℃, but transformants of CH117 with pCHX1 grew well on YEPD at 37℃ (Fig. 1) and showed same drug-sensitivities as the parent (Fig. 2). This indicates a single mutation would be responsible for dhs and ts and pCHX1 would carry the wild type allele of the mutated gene.

The restriction map strategy of the complementary gene as shown in Fig. 8. *Bam*HI-*Bam*HI 3.6 kb cloned in the multicopy vector, pYES2.0. Serial deletion and ligation analysis is shown in Fig.8-A. Deletions of the right *Bam*HI-*Xho*I (0.6 kb) fragment, pCHX2 (3.0 kb) and left *Bam*HI-*Puv*II (0.5 kb), right *Bam*HI-HindIII (1.2 kb) fragment, pCHX5 (1.9 kb) complemented the mutations of CH117 whereas the deletions of left *Bam*HI-*Eco*RI fragment (0.9 kb), pCHX3 (2.7 kb) and right

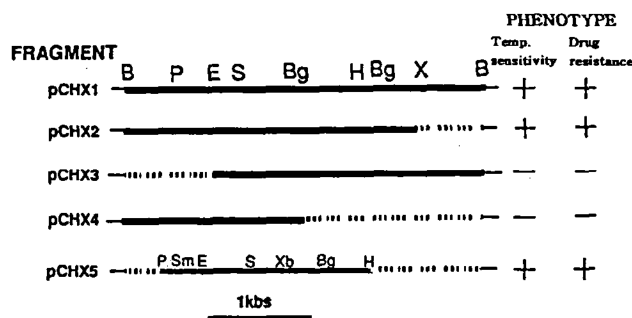

Fig. 6. Restriction nuclease map of wild-type genomic DNA fragment. The original isolated yeast genomic library DNA fragment *Bam*HI-*Bam*HI 3.6 kb, pCHX1 and the essential region *Puv*II-*Hind*III fragment of pCHX5 cloned in pYES2.0 were analyzed by deletion and subcloning. Drug indicates recovery from drug-hypersensitive phenotype assayed by sensitivity to 100 ng/ml cycloheximide at 30℃. Plus of temperature means growth of transformants at 37℃. Abbreviations: B, *Bam*HI; P, *Puv*II; Sm, *Sma*I; E, *Eco*RI; S, *Sal*I; Xb, *Xba*I; Bg, *Bgl*II; H, *Hin*dIII; X, XhoI.

*Bam*HI-*Bgl*II (1.7 kb) fragments, pCHX4 (1.9 kb) did not. Thus the essential region is included in the 1.9kb *Puv*II-*Hind*III fragment in pCHX5.

Transformants of CH117 with pCHX5 showed the same drug-sensitivities as the parent and grew well on YEPD at both 30℃ and 37℃. In conclusion, 1.9 kb *Puv*II-*Hind*III fragment in this plasmid should be essential for complementation. The integrant of pCHX5 gene in the yeast can complement the *dhs* and *ts* phenotype. As shown Fig. 3 and Fig. 4, the transformant pCHX5 also can complement the secretion of periplasmic enzymes such as invertase and acidic phosphatase. Drug-hypersensitivity to various antibiotics with different target is likely due to the defective deposition of cell wall components by mutagenesis. This kind of mutant analysis would be useful for antibiotic treatment of yeast strains including *Candida* spp. The cloned gene appears to be involved in formation of cell wall components and may play a role in this complementation. Further biochemical studies and sequencing of this mutant should reveal the primary action mechanism of cell wall composition for the abnormalities observed in this mutant type.

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