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Diphtheria Toxin-Resistant Mutants of *Saccharomyces cerevisiae*

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We developed a selection procedure based on the observation that diphtheria toxin kills spheroplasts of *Saccharomyces cerevisiae* (Murakami et al., Mol. Cell. Biol. 2:588–592, 1982); this procedure yielded mutants resistant to the in vitro action of the toxin. Spheroplasts of mutagenized *S. cerevisiae* were transformed in the presence of diphtheria toxin, and the transformed survivors were screened in vitro for toxin-resistant elongation factor 2. Thirty-one haploid ADP ribosylation-negative mutants comprising five complementation groups were obtained by this procedure. The mutants grew normally and were stable to prolonged storage. Heterozygous diploids produced by mating wild-type sensitive cells with the mutants revealed that in each case the resistant phenotype was recessive to the sensitive phenotype. Sporulation of these diploids yielded tetrads in which the resistant phenotype segregated as a single Mendelian character. From these observations, we concluded that these mutants are defective in the enzymatic steps responsible for the posttranslational modification of elongation factor 2 which is necessary for recognition by diphtheria toxin.

Diphtheria toxin kills cells by inhibiting protein synthesis through ADP ribosylation of elongation factor 2 (EF-2) (7, 21). The ADP-ribose is attached to EF-2 via a modified histidine residue, termed diphthamide (24), which has been found only in this protein (8, 22). This unique amino acid, 2-[3-carboxyamido-3(trimethylammonio)propyl]histidine (2, 25), is synthesized posttranslationally (26) and occurs within a highly conserved amino acid sequence (6) in the EF-2 of all eucaryotes and archaebacteria (22). The biological role of this amino acid is unknown. To investigate the role of diphthamide, we have sought yeast mutants defective in the posttranslational modification system responsible for its synthesis.

Diphtheria toxin-resistant animal culture cells have been selected and recently have been shown to result either from alterations in the cellular membrane or from alterations in the intracellular target of the toxin, EF-2 (11, 12, 17, 19). Moehring and co-workers (16) have further demonstrated that alterations in EF-2 may be caused by mutations either in the structural gene for EF-2 or in the genes coding for the components of the posttranslational modification system of EF-2 that directs the biosynthesis of diphthamide.

We chose to obtain diphtheria toxin-resistant mutants of the yeast *Saccharomyces cerevisiae* because of the ease of carrying out both genetic and biochemical analyses with this organism. Our previous work (20) showed that yeast spheroplasts are sensitive to intoxication by diphtheria toxin. In this study, we found that some yeast spheroplasts which survive plasmid transformation in the presence of diphtheria toxin possess a toxin-resistant EF-2.

MATERIALS AND METHODS

Yeast strains and growth media. *S. cerevisiae* strain 3482-16-1 (*MATα leu2-3,112 his3Δ1 trp1-289 ura3-52 met2 cyh2*; obtained from L. Hartwell, University of Washington, Seattle) and strain SSL117 (*MATα leu2-3,112 his3Δ1 trp1-289 met2 arg4*; strain collection of D.M.L.) were used in this study. YEPD medium (9), synthetic complete medium (9), YM-1 medium (13), and regeneration medium (20) have been described previously.

Plasmid preparation. Plasmid YEp13 has been described previously (5). This plasmid was isolated from *Escherichia coli* by the alkaline lysis procedure (4).

Ethyl methanesulfonate mutagenesis. Mutagenesis with ethyl methanesulfonate was carried out as described previously (9). Typically, a 10-ml culture of exponentially growing yeast cells was treated with 0.3 ml of the mutagen in 10 ml of 0.1 M phosphate buffer (pH 8.0) for 50 min at 30°C. The level of cell survival under these conditions was 5 to 10%. After mutagenesis the cells were washed and diluted into 10 to 20 separate 10-ml portions of YM-1 complete medium and grown for five or six generations prior to selection.

Yeast transformation and diphtheria toxin treatment. Yeast spheroplasts were transformed by a modification of the procedure of Beggs (1). All steps were performed at 25°C unless otherwise specified. After mutagenesis and growth, 10^8 cells were washed with a solution containing 1.2 M sorbitol, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10 mM Tris hydrochloride (pH 7.5) and then suspended in 1 ml of this solution. Zymolyase 60000 (0.5 mg; Kirin Brewery, Tokyo, Japan) was added, and the cell suspension was incubated for 20 min. The spheroplasts were washed three times with 1.2 M sorbitol and suspended in 0.1 ml of a solution containing 1.2 M sorbitol, 10 mM $CaCl_2$, and 10 mM Tris hydrochloride (pH 7.5). Plasmid YEp13 DNA was added (final concentration, 10 μg/ml), and the suspension was incubated for 15 min. Next, diphtheria toxin was added (final concentration, 200 μg/ml), and after 1 h the preparation was diluted with 1 ml of a solution containing 20% polyethylene glycol 4000, 10 mM $CaCl_2$, and 10 mM Tris hydrochloride (pH 7.5) and incubated for an additional 15 min. The entire spheroplast preparation was mixed with 8 ml of molten regeneration medium lacking leucine, and the mixture was poured onto a synthetic complete plate lacking leucine. Surviving colonies developed in 3 days at 30°C.

Diphtheria toxin and ADP ribosylation of EF-2. Diphtheria toxin was obtained from Connaught Laboratory Ltd., Willowdale, Ontario, Canada. The toxin was dialyzed against a solution containing 1.2 M sorbitol, 1 mM EDTA, and 10 mM Tris hydrochloride (pH 7.5) and was filter sterilized before use in the selection procedure. The toxin sensitivity of EF-2

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in yeast strains was determined by measuring the toxin-catalyzed ADP ribosylation of postmitochondrial extracts. Cultures (10 ml) of exponentially growing cells were washed and suspended in 0.2 ml of a solution containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid], and 5 mM β-mercaptoethanol. Zymolyase 60000 (0.5 mg/ml) was added, and the preparation was incubated for 30 min at 25°C. The resulting cell lysate was centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was collected and subjected to diphtheria toxin-catalyzed ADP ribosylation as described previously (23). The reaction was initiated by adding 10 μl of cell extract (usually containing 0.05 to 0.10 mg of protein) to 40 μl of reaction mixture containing 20 mM Tris hydrochloride (pH 7.5), 50 mM dithiothreitol, 1 mM EDTA, 10 μM [*adenylate*-^{32}P]NAD$^+$ (ca. 10^6 cpm; New England Nuclear Corp., Boston, Mass.), and 0.2 μg of diphtheria toxin. The solution was incubated at 37°C for 15 min, and the reaction was terminated by adding 50 μl of 20% (wt/vol) cold trichloroacetic acid. The precipitate was collected on a cellulose nitrate membrane filter (pore size, 0.45 μm; diameter, 25 mm; Micro Filtration Systems), washed with 5% (wt/vol) cold trichloroacetic acid, and subjected to liquid scintillation counting after solubilization of the filter in 95% Aquasol II (New England Nuclear Corp.). We previously demonstrated that under these conditions only EF-2 is labeled (23).

Genetic analysis. Tetrad analysis was performed by crossing each of the mutants with a wild-type strain, sporulating the resulting diploids, and dissecting the tetrads. The phenotypes of the spores were analyzed by measuring the ADP-ribose acceptor activity of extracts prepared from each progeny strain. A tester strain of each mutant was prepared by scoring the spores for both toxin resistance and the desired mating type. Complementation analysis was carried out by crossing each of the mutants with appropriate tester strains and assaying for toxin resistance in extracts of the resulting diploid cells. After we identified heterozygous diploids which were previously formed for sporulation and dissection and had lost plasmid DNA, the dominance characteristics of the mutations were determined by again subjecting the mutants to plasmid transformation in the presence of diphtheria toxin.

RESULTS

Rationale for the selection of diphtheria toxin-resistant yeast mutants. Our previous study (20) showed that yeast spheroplasts are sensitive to the action of diphtheria toxin, while intact cells are not, presumably because the yeast cell wall blocks toxin-binding sites on the cell membrane. The sensitivity of yeast spheroplasts to toxin suggested the possibility of selecting toxin-resistant yeast cells by subjecting spheroplasts to toxin and recovering the survivors. Unfortunately, we found that intact cells, which escaped spheroplast formation and were resistant to intoxication, constituted approximately 1 to 10% of our spheroplast preparations. To eliminate this subpopulation of intact cells, survivors of diphtheria toxin treatment were recovered under conditions in which only those cells converted to spheroplasts constituted the experimental population. This was accomplished by simultaneous selection for cells transformed with a plasmid since spheroplats, but not intact cells, were transformed under the conditions used. The inhibitory effects of various toxin concentrations on the recovery of transformed yeast spheroplasts are shown in Fig. 1. In the absence of toxin, YEp13 transformed strain 3482-16-1 at an efficiency of 10^3 to 10^4 transformants per μg of DNA. With increasing diphtheria toxin concentrations, significant killing of the transformed spheroplasts was observed. Maximum killing (>99.9%) was achieved by a 15-min treatment with diphtheria toxin at a concentration of 20 μg/ml.

Based on the observation that diphtheria toxin can efficiently kill spheroplasts which are capable of being transformed by plasmid DNA in the presence of $CaCl_2$, we constructed a selection procedure which directly yielded the toxin-resistant mutants of interest. The scheme used for isolation of diphtheria toxin-resistant mutants is shown in Fig. 2. Yeast spheroplasts prepared from mutagenized cells were transformed with plasmid DNA and then treated with diphtheria toxin. A 1-h treatment with 200 μg of diphtheria toxin per ml was used routinely. After selection on appropriate agar plates, the surviving colonies which developed within 3 days at 30°C were subjected to further screening. Typically, a comparison of the number of survivors after transformation in the presence of toxin with the number of survivors after control transformations showed that less than 0.1% of the transformants escaped killing by toxin.

Screening of diphtheria toxin-resistant mutants. To obtain mutants in which toxin resistance resulted from an alteration in EF-2 rather than an alteration in the membrane, the transformants which survived the selection procedure were screened in vitro for toxin-resistant EF-2. Postmitochondrial extracts prepared from each surviving colony were assayed for ADP-ribose acceptor activity of EF-2 in the presence of diphtheria toxin and [^{32}P]NAD$^+$. The level of labeling of wild-type yeast extracts ranged from 30 to 50 pmol of ADP-ribose incorporated per mg of protein. Strains with less than 10% of the normal ADP-ribose acceptor activity were scored as diphtheria toxin-resistant mutants in which EF-2 was refractory to diphtheria toxin-catalyzed inactivation. Thirty-one independent isolates of this type of toxin-resistant mutant were obtained by this procedure from 69 independent survivors. To eliminate the possibility that low levels of ADP-ribose acceptor activity were caused by components in the extracts which interfered with the toxin-

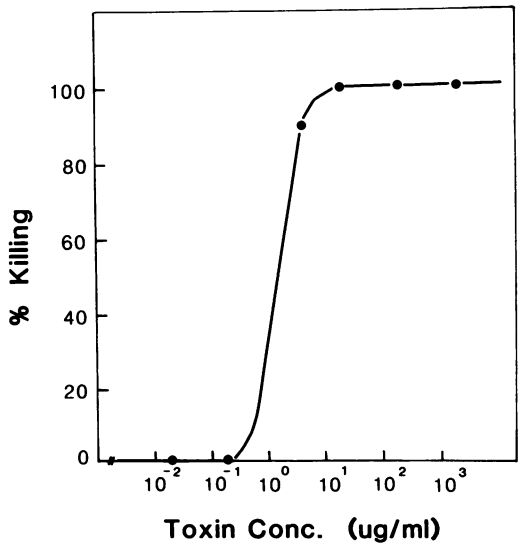

FIG. 1. Killing efficiency of different concentrations of diphtheria toxin on transformed spheroplasts of strain 3482-16-1.

catalyzed ADP ribosylation reaction, purified wild-type EF-2 was incubated with each of the extracts during the ADP ribosylation assay. The results showed that the ADP-ribose acceptor activity of the added wild-type EF-2 was not depressed by any of the extracts (data not shown).

Genetic characterization of toxin-resistant mutants. Tetrad analysis was carried out with many of the mutant isolates, including at least one member of each complementation group (see below). Each mutant was crossed with a toxin-sensitive strain of the opposite mating type, the resulting diploids were sporulated, and the spore clones were analyzed by the toxin-catalyzed ADP ribosylation assay. On average, five tetrads were analyzed for each cross. Representative data from one such cross are shown in Table 1. In each case, the toxin-resistant phenotype segregated 2:2, as expected of a single chromosomal mutation.

The susceptibility to toxin of diploids heterozygous for the resistance trait was examined by using the spheroplast-plasmid transformation procedure (data not shown). Spheroplasts of all of the heterozygous diploids were found to be sensitive to the action of diphtheria toxin. These results demonstrated that the resistance phenotype is recessive.

To place the mutant isolates into complementation groups, alpha mating type tester strains were mated in pairwise combinations with the 31 recessive toxin-resistant mutants, and the diploids were analyzed by the toxin-catalyzed ADP ribosylation assay. Five genetic complementation groups were found, and they were designated *dph1*, *dph2*, *dph3*, *dph4*, and *dph5*; these complementation groups contained 17, 9, 1, 3, and 1 members, respectively.

TABLE 1. Segregation of the diphtheria toxin-resistance phenotype in tetrads[a]

Strain	ADP-ribose incorporation (pmol/mg of protein)
3482–16–1	34.7
JC3.3.2	2.4
1a	2.2
1b	30.7
1c	30.5
1d	0.8
2a	3.9
2b	53.6
2c	7.0
2d	42.5
3a	1.4
3b	27.9
3c	36.8
3d	3.1
4a	35.2
4b	1.4
4c	3.1
4d	29.9
5a	2.4
5b	34.0
5c	1.6
5d	27.8

[a] Diploids formed by mating strains JC3.3.2 (*dph2*) and 3482–16–1 were subjected to sporulation. Ascospores were dissected, and the phenotypes of the tetrads were analyzed by measuring the levels of ADP-ribose incorporation by extracts after incubation with diphtheria toxin and [^{32}P]NAD$^+$.

DISCUSSION

We developed a selection scheme which yielded yeast mutants resistant to the action of diphtheria toxin. To select such mutants, we applied toxin to spheroplasts during a plasmid DNA transformation procedure and collected the few transformed survivors. Some of these isolates possessed EF-2 which was resistant to in vitro ADP ribosylation by diphtheria toxin. Characterization of the mutants revealed that they have single, recessive chromosomal defects which comprise five complementation groups.

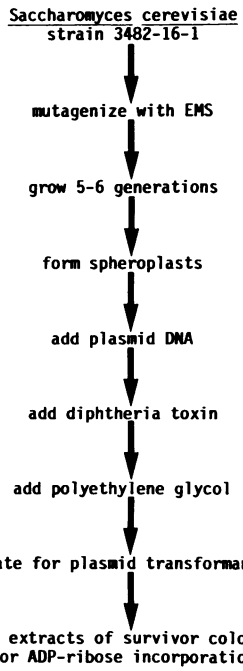

FIG. 2. Selection and screening scheme used for diphtheria toxin-resistant yeast mutants. EMS, Ethyl methanesulfonate.

Two types of diphtheria toxin-resistant mutants of Chinese hamster ovary cells in which EF-2 is altered have been described (16). The first type results from mutations in the genes directing the posttranslational biosynthesis of diphthamide from a histidine residue encoded in the EF-2 gene, and the second type results from mutations in the EF-2 gene itself. The mutations of the first type are recessive, while the mutations of the second type are codominant in Chinese hamster ovary cells (17). Based on these results with mammalian cells, the recessive nature of the mutations of the toxin-resistant yeast mutants described in this paper suggests that the yeast mutants do not have an altered EF-2 gene but rather have a defect in one of the genes coding for the enzymes involved in the posttranslational synthesis of diphthamide. None of the mutants appeared to have any growth defects, such as temperature-dependent growth. Therefore, we conclude that yeast cells, like mammalian cells (16), do not require diphthamide-containing EF-2, at least for growth under the conditions which we used.

We considered two possible explanations for our apparent failure to isolate mutants with a mutation in the EF-2 gene. First, a toxin-resistant mutation in the EF-2 gene is probably rarer than mutations affecting the modification system. A toxin-resistant EF-2 gene mutant would have to produce an altered yet functional EF-2, while a mutant with a similar mutation in a gene responsible for modification need only yield an inactive product. Second, *S. cerevisae* has more

than one copy of the EF-2 gene, mutations affecting all copies (and resulting in a complete absence of ADP-ribosylatable EF-2) would be rare. Some yeast ribosomal protein genes are known to be duplicated (3, 10, 27), as are certain other genes encoding essential, abundant proteins (14, 15). Duplication of the EF-2 gene in yeast cells would contrast with the situation in mammalian cells, which appear to have only a single gene copy (17). However, some of the survivors of the selection scheme described in this paper appeared to exhibit intermediate levels of ADP ribosylation. These strains have not been investigated further, but if the EF-2 gene is duplicated in yeast cells, some of these strains may include EF-2 gene mutations.

In our previous work (20) we presented evidence which suggested that diphtheria toxin requires a specific membrane receptor for penetration into the cytoplasm of yeast spheroplasts. However, two observations suggest that the penetration of toxin into yeast spheroplasts undergoing plasmid transformation in the presence of Ca^{2+} does not require the participation of a receptor. First, the rate of toxin killing of spheroplasts undergoing transformation (data not shown) appears to be more rapid than the rate of killing of spheroplasts in the absence of transformation (20). Second, we have recently observed that the catalytic subunit of another toxin, ricin, kills yeast spheroplasts undergoing transformation in the complete absence of the membrane-binding subunit of ricin (data not shown). Thus, although further work will be needed to clarify the mechanism(s) involved in the penetration of protein toxins into yeast spheroplasts, we believe that the procedure described here may be applicable to the selection of yeast mutants resistant to other normally impenetrable inhibitors.

Mutants with mutations in diphthamide biosynthesis provide a basis for biochemical analysis of both the functional role and biosynthetic pathway of this amino acid. In this regard, we have recently observed that the EF-2 found in *dph5* mutants can be converted into an ADP-ribose-accepting form by an enzyme present in the other *dph* mutants and in wild-type yeast cells. Thus, the yeast *dph5* mutant, like the CG-1 mutant of Chinese hamster ovary cells described by Moehring et al. (18), appears to correspond to an enzyme which acts late in the pathway of diphthamide biosynthesis.

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