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DPH5, a Methyltransferase Gene Required for Diphthamide Biosynthesis in *Saccharomyces cerevisiae*

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A mutant of *Saccharomyces cerevisiae* defective in the *S*-adenosylmethionine (AdoMet)-dependent methyltransferase step of diphthamide biosynthesis was selected by intracellular expression of the F2 fragment of diphtheria toxin (DT) and shown to belong to complementation group *DPH5*. The *DPH5* gene was cloned, sequenced, and found to encode a 300-residue protein with sequence similarity to bacterial AdoMet: uroporphyrinogen III methyltransferases, enzymes involved in cobalamin (vitamin B_{12}) biosynthesis. Both DPH5 and AdoMet:uroporphyrinogen III methyltransferases lack sequence motifs commonly found in other methyltransferases and may represent a new family of AdoMet:methyltransferases. The *DPH5* protein was produced in *Escherichia coli* and shown to be active in methylation of elongation factor 2 partially purified from the *dph5* mutant. A null mutation of the chromosomal *DPH5* gene did not affect cell viability, in agreement with other studies indicating that diphthamide is not required for cell survival. The *dph5* null mutant survived expression of three enzymically attenuated DT fragments but was killed by expression of fully active DT fragment A. Consistent with these results, elongation factor 2 from the *dph5* null mutant was found to have weak ADP-ribosyl acceptor activity, which was detectable only in the presence of high concentrations of fragment A.

The diphthamide residue of elongation factor 2 (EF-2), a modified histidine {2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine} (49), represents an intriguing example of posttranslational modification of proteins. Both diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A catalyze transfer of the ADP-ribose moiety of NAD specifically to the diphthamide residue of EF-2, inactivating the factor and causing a lethal blockage of protein synthesis (9). Diphthamide is present in one equivalent per mol of EF-2 (and only EF-2) in all eukaryotic organisms examined (12) but has not been found in eubacteria. There is also evidence of diphthamide, or a precursor of diphthamide, in archaebacteria (35). While the function of diphthamide in toxin action is well established, the residue's role in cellular physiology remains obscure. The study reported here represents a step towards understanding the biosynthesis and function of diphthamide in greater detail.

Mutant cells defective in diphthamide biosynthesis have been selected in other laboratories by treating toxin-sensitive cells with either DT or exotoxin A. Mutants of Chinese hamster ovary (CHO) cells, isolated by Moehring and coworkers, were found to be recessive and comprised three complementation groups (30, 31). Similar mutants in *Saccharomyces cerevisiae*, selected by exposing spheroplasts to DT, were also recessive and comprised five complementation groups (*dph1*, *dph2*, *dph3*, *dph4*, and *dph5*) (8). Diphthamide mutants isolated in this manner apparently grow at an undiminished rate, and no distinctive phenotype besides toxin resistance has been ascribed to them (8, 23, 30).

A biosynthetic pathway for diphthamide has been proposed on the basis of labelling studies and biochemical analysis of wild-type and toxin-resistant cells (Fig. 1). Labelling studies with yeast suggest that diphthamide biosynthesis begins with a rare 3-amino-3-carboxypropyl transfer from *S*-adenosylmethionine (AdoMet) to the imidazole C-2 of the precursor histidine residue in EF-2 (12). Trimethylation of the resulting amino group follows, with AdoMet as the methyl donor, to produce diphthine 3 (7, 29). The final step is an ATP-dependent amidation of the donated carboxyl group, yielding diphthamide 4 (7, 31).

Despite the availability of various diphthamide mutants, none of the genes of the diphthamide biosynthetic pathway has been isolated heretofore. We now report the isolation and sequencing of one such gene from *S. cerevisiae*. Evidence that the protein product of this gene, *DPH5*, catalyzes the methylation step of diphthamide biosynthesis is presented.

MATERIALS AND METHODS

Strains, genetic methods, and manipulations of yeast strains. All *S. cerevisiae* strains used or constructed in this work are derived from the laboratory collection of Fred Winston, Harvard Medical School, and are derivatives of strain S288C. Standard methods for mating, sporulation, and tetrad analysis were used (2, 45). Rich medium (YPD) and minimal medium (SD) were supplemented with relevant amino acids and 2% glucose or galactose as a carbon source (45). Sporulation medium was prepared as described previously by Sherman et al. (45), and GNA presporulation medium contained 10 g of yeast extract, 30 g of nutrient broth, 50 g of glucose, and 20 g of agar per liter. Growth rates were determined by measuring the optical density at 600 nm. For all experiments, yeast cells were grown at 30°C.

Preparation of yeast extracts. Extracts were prepared as described elsewhere (39), with modifications. Cells were grown to an optical density at 600 nm of approximately 1, harvested by centrifugation, and washed with 1 ml of EB buffer (200 mM Tris-HCl [pH 8.0], 400 mM ammonium sulfate, 10 mM magnesium chloride, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 7 mM

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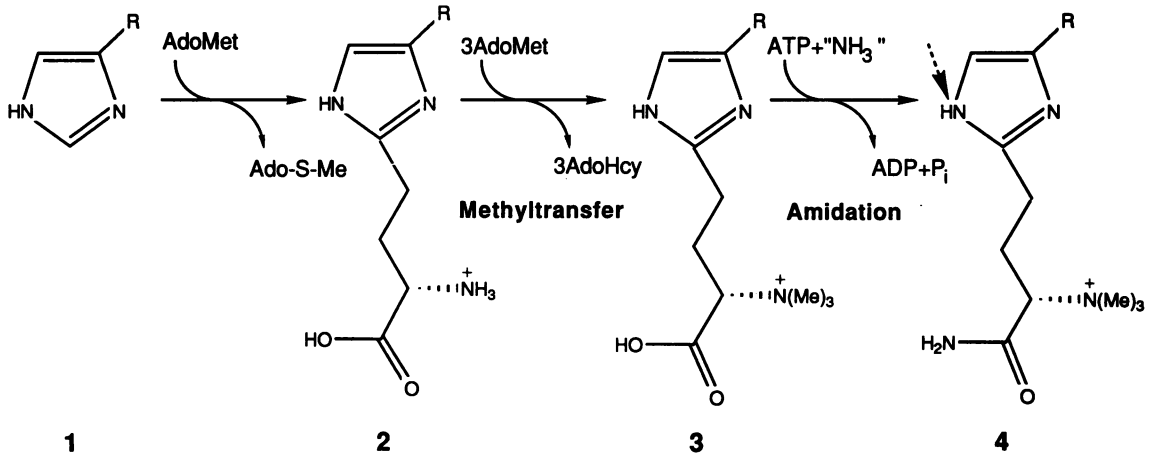


FIG. 1. Proposed pathway of diphthamide biosynthesis (7, 30). The numbers below the structures refer to precursors described in the text. Structures 3 and 4 have been given the trivial names of diphthine and diphthamide, respectively (49). The dashed arrow indicates the imidazole nitrogen that is ADP-ribosylated by DT.

β-mercaptoethanol). The cell pellet was resuspended in 0.4 ml of EB buffer and vortexed four times (1-min intervals) at 4°C in the presence of 0.3 g of acid-washed glass beads (0.45-mm diameter). The extracts were centrifuged for 15 min at 10,000 × *g*, and the supernatants were collected and stored at −70°C. Protein concentrations were determined with Coomassie blue G-250 reagent (Pierce, Rockford, Ill.).

Biochemical complementation of ADP-ribosyl acceptor activity. Strains FY2, LMY10, and LMY11 were grown in 20 ml of YPD, and extracts were prepared as described above. Extracts were desalted by passage over a G-50 spun column equilibrated with 50 mM Tris-HCl [pH 7.5], 5 mM β-mercaptoethanol, and 1 mM EDTA. The reaction buffer included an energy-generating system as previously described (7) and contained 80 mM Tris-HCl [pH 7.5], 5 mM magnesium acetate, 15 mM potassium chloride, 5 mM β-mercaptoethanol, 1 mM ATP, 10 mM creatine phosphate, 80 μg of phosphocreatine kinase per ml, 10 μM AdoMet, and 10 μM glutamine in a final volume of 40 μl. Each reaction contained 26 μg of total protein (13 μg of protein from each extract) and was incubated for 4 h at 30°C. Afterwards, the reactions were desalted on a G-50 spun column and tested for ADP-ribosyl acceptor activity in the presence of 136 nM [*adenine*-^{3}H]NAD (29.3 Ci/mmol; New England Nuclear) and 590 nM DT fragment A (see below).

Methyltransferase assay. Buffer conditions were the same as for biochemical complementation of ADP-ribosyl acceptor activity, except that AdoMet and glutamine were replaced with 210 μM [*methyl*-^{3}H]AdoMet (12.4 Ci/mmol; New England Nuclear). After a 1.5-h incubation at 30°C, a portion of the reaction was solubilized in sodium dodecyl sulfate (SDS) sample buffer and proteins were resolved by electrophoresis on an SDS–7.5% polyacrylamide gel (25). Labelled proteins were detected by autoradiography. To test for labelling of EF-2 by transfer of the 3-amino-3-carboxypropyl group from AdoMet, the ^{3}H-labelled AdoMet was replaced with [*carboxyl*-^{14}C]AdoMet (53 mCi/mmol; Amer-

TABLE 1. Yeast strains

Strain	Genotype	Source
FY2	*MATα ura3-52*	F. Winston
FY69	*MATa leu2Δ1*	F. Winston
LMY10	*MATα ura3-52 dph5*	This work
LMY11	*MATα ura3-52 dph2*	This work
LMY12	*MATa ura3-52 leu2Δ1 dph5*	This work
LMY14	*MATα ura3-52 trp1Δ63 dph5*	This work
LMY15	*MATα ura3-52 trp1Δ63 dph2*	This work
0407	*MATa leu2-3,112 his3Δ1 trp1-289 ura3-52 met2 cyh2 dph5*	J. W. Bodley
LMY16	*MATα ura3-52 trp1Δ63*	This work
LMY20	*MATa/MATα ura3-52/ura3-52 leu2Δ1/LEU2 TRP1/trp1Δ63 dph5/DPH5*	This work
LMY23	*MATa/MATα leu2Δ1/LEU2 URA3/ura3-52 DPH5/dph5*	This work
LMY28	*MATa ura3-52 leu2Δ1*::toxin-*LEU2 dph5*	This work
LMY36	*MATa/Matα ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/TRP1 HIS3/his3Δ200*	This work
LMY37	*MATa/MATα dph5::LEU2/DPH5 ura3-52/ura3-52 leu2Δ1/ leu2Δ1 trp1Δ63/TRP1 HIS3/his3Δ200*	This work
LMY38	*MATα ura3-52 leu2Δ1 trp1Δ63*	This work
LMY39	*MATa dph5::LEU2 ura3-52 leu2Δ1 his3Δ200*	This work
LMY41	*MATα ura3-52 trp1Δ63* [*DPH5*-pLMY214-*URA3*]	This work

sham) or [*methyl*-^{14}C]AdoMet (43 mCi/mmol; New England Nuclear) at a concentration of 116 μM.

ADP-ribosyl acceptor activity. ADP-ribosylation of EF-2 was assayed in a final volume of 100 μl containing 50 mM Tris-HCl [pH 8.2], 1 mM EDTA, 40 mM dithiothreitol, and radiolabelled NAD and purified DT fragment A (5), as indicated in the figure legends. After a 1-h incubation at 30°C, the reaction was terminated by applying duplicate samples to trichloroacetic acid-impregnated paper (6) and the samples were counted by liquid scintillation.

Plasmids. Plasmid pLMY101 was constructed by ligating a 1.2-kb *Bam*HI fragment encoding the F2 fragment of DT to the yeast 2μm plasmid, pRY131 (52). The 1.2-kb fragment was isolated from ptacF2 (50) in which the *Hin*dIII site of ptacF2 was first converted to *Bam*HI with the Klenow fragment and the linker d(CGCGGATCCGCG). Plasmid pLMY109 was constructed by subcloning the 4-kb *Xho*I-*Sac*I fragment from pLMY101 to a derivative of the yeast integrative plasmid YIp351 (18) in which the *Sma*I site of YIp351 was first converted to *Xho*I with the Klenow fragment and the linker d(CCCTCGAGGG). Plasmid pLMY215, containing the *DPH5* gene under the transcriptional control of the bacteriophage T7 promoter, was constructed by first subcloning the 1.2-kb *Eco*RI-*Sac*II fragment of pLMY198 to the *Eco*RI-*Sma*I sites of M13mp18 (51). By oligonucleotide-directed in vitro mutagenesis (44), an *Nde*I site spanning the *DPH5* translation start codon (Met-1; see Fig. 5) was created with the oligonucleotide d(AATAAAGCATATG TCTCACT), yielding M13LM106. The replicative form of M13LM106 was isolated, and the 1.2-kb *Nde*I-*Bam*HI fragment was subcloned to the same sites of the T7 promoter plasmid pT7-7 (2).

Transformations and DNA isolation. Yeast cells were transformed by the lithium acetate method (21). *Escherichia coli* HB101 (4) and XL1-Blue (Stratagene, La Jolla, Calif.) were transformed by standard techniques (2). Plasmid DNA from yeast was isolated by the plasmid rescue method (19), and plasmid DNA from *E. coli* was isolated as previously described (2). Genomic DNA from yeast was isolated from spheroplasts as previously described (2).

Deletion analysis of the *DPH5* gene and DNA sequencing. The 2.4-kb *Sph*I-*Bam*HI genomic fragment that complements the *dph5* mutant is carried by plasmid pLMY117, an *Sph*I deletion derivative of the YCp50 library plasmid pLMY111. The *Sph*I site of pLMY117 was converted to *Eco*RI with synthetic linkers to yield pLMY128. Plasmid pLMY128 was restricted with *Eco*RI (a second vector *Eco*RI site flanks the *Bam*HI site), and the 2.4-kb *Eco*RI fragment was subcloned in both orientations to the *Eco*RI site of pBluescript pKS (Stratagene), yielding pLMY136 and pLMY137. Unidirectional, nested deletions of pLMY136 and pLMY137 were by the exonuclease III (ExoIII)-mung bean system (Stratagene) after restriction at a unique *Sma*I site on the vector. Deletion subclones were sequenced and tested for complementation by restricting at the *Eco*RI and vector *Sac*II sites and subcloning the deletion fragments to the same sites of pRS316 (46). Double-stranded sequencing was by the method of Sanger et al. (43) using ^{35}S-dATP (Amersham) and the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). Computer analysis of the nucleotide and protein sequence data was done by using the Genetics Computer Group version 6.0 (11) and the Pustell sequence analysis software (IBI, New Haven, Conn.).

Construction of *dph5* null mutant. Plasmid pLMY210 was constructed for the disruption of *DPH5* as follows. The 1.2-kb *Eco*RI-*Sac*II restriction fragment from pLMY198 containing the *DPH5* coding sequence (see Fig. 4) was subcloned to a derivative of pKS in which the *Hin*dIII site of pKS was destroyed by filling in with the Klenow fragment and religating. To construct the *dph5* null allele, *dph5*::*LEU2*, the 0.28-kb *Nco*I-*Hin*dIII restriction fragment internal to *DPH5* was replaced with a 3.1-kb *Hin*dIII-*Aat*II restriction fragment from plasmid pRS305 (46) containing the *LEU2* gene. The resulting plasmid, pLMY212, was cleaved at the unique *Sal*I site and used to transform LMY36 to leucine prototrophy. Strain LMY37 is a stable Leu$^+$ transformant resulting from recombination between the plasmid and the genome, such that one copy of the *DPH5* gene was replaced by the null allele. The structure of the *DPH5* loci of strain LMY37 was verified by Southern blot hybridization analysis using nitrocellulose as described elsewhere (27). The probe used for hybridization was labelled with [α-^{32}P]dCTP by the random primer method (14).

Partial purification of EF-2 from the *dph5* null mutant. Strain LMY39 was grown in 500 ml of YPD. At an optical density at 600 nm of 3, cells were harvested, resuspended in 40 ml of cold EB buffer, and lysed by French press. Cell debris were removed by centrifugation at 30,000 × *g* for 20 min. The resulting supernatant was adjusted to 0.5 M KCl and centrifuged for 2.5 h at 45,000 rpm in a Beckman 70.1 Ti rotor to pellet ribosomes. Proteins from the ribosome-free supernatant were precipitated by addition of ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation, resuspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (20 mM HEPES [pH 7.5], 0.1 mM EDTA, 6 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and dialyzed against the same buffer overnight. Approximately 1.5 mg of protein was applied to a Mono Q HR 5/5 anion-exchange column (Pharmacia LKB Biotechnology Inc.) equilibrated with HEPES buffer, and proteins were eluted with a linear KCl gradient (0 to 1 M). Fractions (0.5 ml) were collected, and proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). EF-2-containing fractions were determined by comigration with a purified EF-2 standard and assayed separately for ADP-ribosyl acceptor and methyl acceptor activities by using 20- and 5.5-μl samples, respectively. ADP-ribosyl acceptor activity was determined as described in the legend to Fig. 9, and labelled EF-2 bands were quantified by scanning (PhosphorImager; Molecular Dynamics, Sunnyvale, Calif.). Methyl acceptor activity was determined as described above by using 10 μg of protein from the recombinant *DPH5* extract, followed by trichloroacetic acid precipitation and liquid scintillation counting.

Nucleotide sequence accession number. The GenBank accession number for the *DPH5* sequence is M83375.

RESULTS

Isolation of toxin-resistant mutants. Yeast spheroplasts, unlike intact yeast cells, are sensitive to the action of exogenously added DT, and this sensitivity was used earlier to isolate toxin-resistant mutants (8). To circumvent the technical difficulties inherent in using spheroplasts to isolate mutants, we instead expressed the gene for an enzymically active DT fragment (F2) within yeast cells. DT is normally secreted as a single polypeptide that is subsequently nicked and reduced to release the A and B fragments. The A fragment contains the catalytic site for ADP-ribosylation, while B is involved in receptor binding and membrane translocation. The F2 fragment, which has reduced enzymic

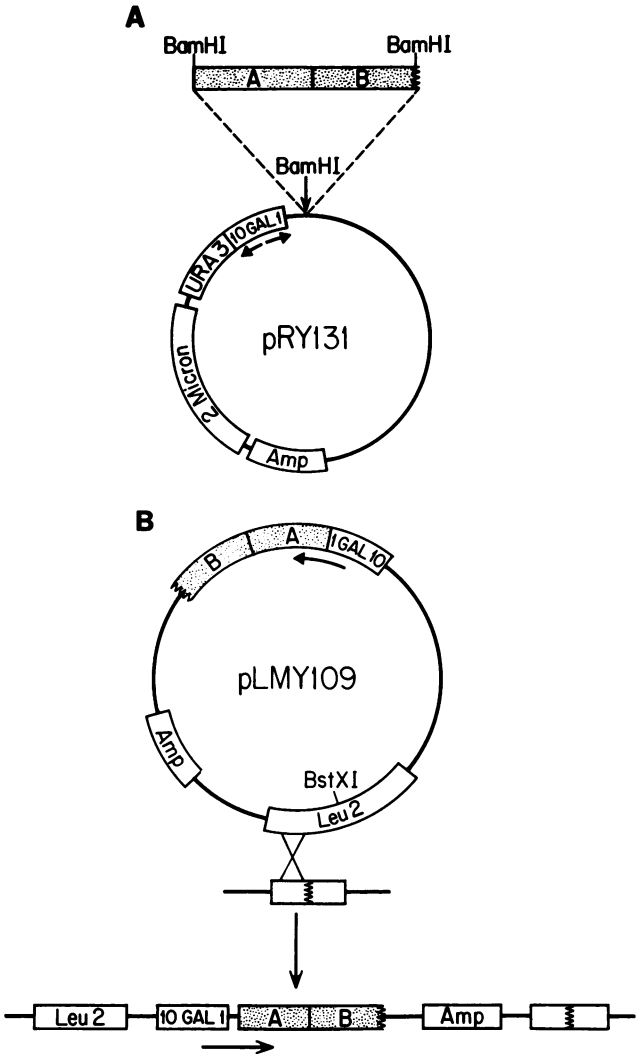


FIG. 2. Construction of plasmids containing the F2 fragment of DT under the yeast *GAL1* promoter and integration of F2 at the *LEU2* chromosomal locus. (A) Construction of plasmid pLMY101. (B) Plasmid pLMY109, containing the F2 toxin gene under *GAL1* control, was linearized at the unique *Bst*XI site within the *LEU2* gene and used to transform strain LMY12 to leucine prototrophy. Stable Leu$^+$ transformants were selected, resulting in strain LMY28.

activity relative to fragment A, comprises the entire A fragment and part of B (48). We placed F2 under the transcriptional control of the yeast *GAL1* promoter, resulting in plasmid pLMY101 (Fig. 2A), and found that strain FY2 carrying this plasmid grew on plates containing glucose as the sole carbon source but was killed on plates containing galactose instead. When unmutagenized FY2 carrying pLMY101 was grown on galactose plates, colonies arose at a frequency of about one in 10^7 cells plated. We tested extracts of 15 survivors for ADP-ribosyl acceptor activity and found 2 (LMY10 and LMY11) that had no detectable activity. The remaining 13 presumably carried either host or plasmid mutations that prevented functional expression of the toxin and were not further characterized.

Biochemical characterization of a toxin-resistant mutant. The absence of ADP-ribosyl acceptor activity in extracts

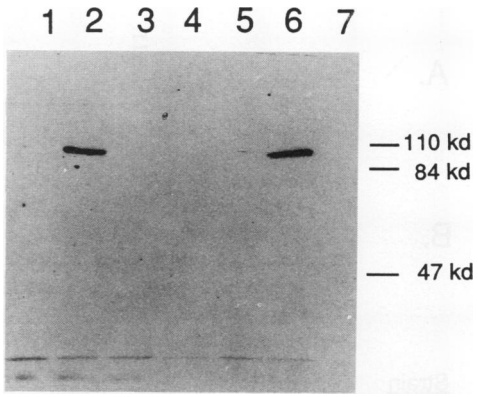
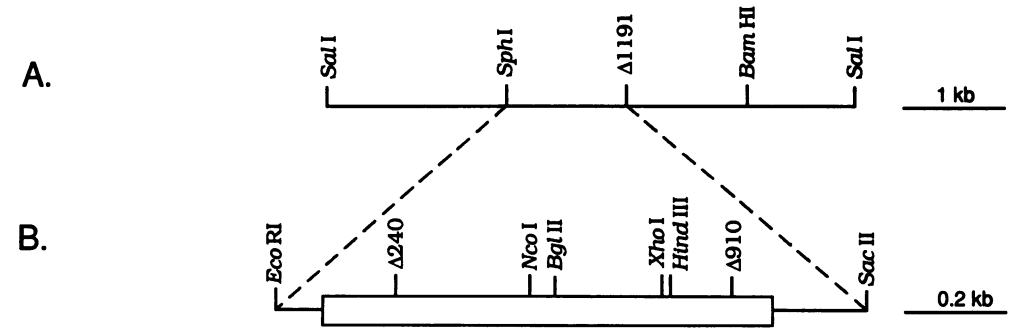


FIG. 3. AdoMet-dependent methyltransferase activity of wild-type and toxin-resistant cells. Shown is an autoradiogram of reaction products resolved by SDS–7.5% PAGE. Reaction conditions are described in Materials and Methods and included 55 μg of total protein from each extract. The following combinations of extracts are shown: lane 1, FY2; lane 2, FY2 plus LMY10; lane 3, FY2 plus LMY11; lane 4, LMY10; lane 5, LMY11; lane 6, LMY10 plus LMY11; lane 7, control, no extract. The locations of molecular mass markers are shown at the right.

prepared from strains LMY10 and LMY11, as determined with DT fragment A under standard assay conditions, suggested that the toxin resistance of the two mutants was caused by lesions in one or more steps of diphthamide biosynthesis or in EF-2 itself. Significant levels of ADP-ribosyl acceptor activity appeared when we combined and incubated the mutant extracts in the presence of an ATP-generating system: no-extract control, 6 fmol of ADP-ribosylated EF-2 per reaction; FY2, 436 fmol; LMY10, 5 fmol; LMY11, 6 fmol; and LMY10 plus LMY11, 65 fmol. This indicates that the mutant strains belong to different complementation groups. The activity of the complemented mutant extracts was lower than that of wild type and may be caused by an inefficient in vitro modification system.

EF-2-specific methyl acceptor activity has been demonstrated in vitro in certain yeast and mammalian cell mutants that are apparently deficient in methyltransferase activity (7, 29). To test for methyl acceptor activity, we incubated each mutant extract with the other or with an extract from wild-type cells and tested for incorporation of label into protein from AdoMet radiolabelled at the methyl group. As shown in Fig. 3, the LMY10 mutant extract contained a protein of 100 kDa, the approximate molecular mass of EF-2 (24), that was labelled in the presence of extracts from wild-type cells (FY2) or the LMY11 mutant. This result suggested that wild-type or LMY11 mutant cells contain a methyltransferase capable of methylating EF-2 in the LMY10 extract. No labelling of EF-2 was detected in wild-type extracts (Fig. 3, lane 1), presumably because wild-type EF-2 is completely methylated and does not serve as a substrate for the enzyme.

It was conceivable that diphthamide biosynthesis in the LMY10 mutant was blocked at an earlier step and that the observed methylation occurred secondarily, after generation of the precursor 2 by 3-amino-3-carboxypropyl transfer in vitro in the complemented extracts. We therefore repeated the methyltransferase assay in the presence of AdoMet containing ^{14}C at the carboxyl or methyl group position. Labelling of EF-2 in the LMY10 extract occurred only in the presence of methyl-labelled AdoMet, indicating that 3-ami-



Strain	Plasmid	ADP-ribosylated EF-2 (fmoles per reaction)
FY69		621
LMY12	pLMY198	608
LMY12	pLMY132	2.7
LMY12	pLMY133	3.6
LMY12	pLMY199	4.3
LMY12	pLMY203	3.0
LMY12	pRY131	2.9

FIG. 4. (A) Restriction map of the *DPH5* locus and location of the ExoIII deletion end point at nucleotide position 1191. Numbering of nucleotides begins at the *Sph*I site. (B) Restriction map and location of ExoIII deletion end points of the *DPH5* gene. The open rectangle represents the predicted coding sequence of the *DPH5* product, with the contiguous lines representing flanking chromosomal and vector sequences. The *Nco*I and *Hin*dIII sites shown were used to create the *dph5* null mutant. The lines below represent the boundries of subclones used to test for complementation. Also listed is corresponding ADP-ribosyl acceptor activity of the wild-type control strain FY69 and *dph5* mutant strain LMY12 after transformation with the various pRS316-derived plasmids. Plasmids pLMY132 and pLMY133 are *Xho*I deletion subclones, and plasmids pLMY199 and pLMY203 contain ExoIII deletions that terminate at nucleotide positions 910 and 240, respectively. Plasmid pLMY198 is described in the text, and plasmid pRY131 is a vector control (52). Strains were grown in 20 ml of SD medium, and extracts were prepared and assayed for acceptor activity as described in Materials and Methods. ADP-ribosyl acceptor assays contained 26 μg of total protein, 30 nM [*adenine*-^{3}H]NAD (33.5 Ci/mmol; New England Nuclear), and 63 nM DT fragment A. A control reaction lacking extract yielded an acceptor activity of 4.1 fmol of ADP-ribosylated EF-2.

no-3-carboxylpropyl transfer did not precede methyl transfer in our assay. These results indicated that the LMY10 mutant contained a lesion in the methyltransferase step of diphthamide biosynthesis.

Genetic characterization of the methyltransferase mutant. When strains LMY12 and LMY15 were crossed, the resultant diploid had ADP-ribosyl acceptor activity equal to that of a wild-type diploid strain (LMY36) and indicated that both strains belong to different complementation groups.

Previous studies have shown that one of the five yeast complementation groups, *DPH5*, functions in the methylation step of diphthamide biosynthesis (7). To determine whether our methyltransferase mutant belonged to the same complementation group, we crossed our mutant strain LMY14 with the *dph5* mutant strain 0407 (8). The resultant diploid had no detectable ADP-ribosyl acceptor activity, indicating that our mutant belonged to the same complementation group (*DPH5*). We also found, by a similar mating assay using *dph1*, *dph2*, *dph3*, and *dph4* mutant strains, that our second diphthamide mutant (LMY11) belongs to complementation group *DPH2* (28).

The *dph5* mutant, LMY10, was crossed with the toxin-sensitive strain FY69 to yield the heterozygous diploid LMY23. Sporulation of LMY23 and tetrad analysis of nine complete tetrads showed that ADP-ribosyl acceptor activity segregated 2:2. Thus, the *dph5* mutation is a single, chromosomal mutation.

To test whether the *dph5* mutation is dominant or recessive, we crossed LMY12 with LMY16 to yield the heterozygous diploid LMY20. Transformation of LMY20 with plasmid pLMY101 gave transformants which grew on plates containing glucose but were killed on galactose-containing plates. Thus, toxin resistance and the *dph5* mutation are recessive, properties that are consistent with those of other mutants defective in diphthamide biosynthesis (8, 30).

Cloning of the *DPH5* gene by complementation. Because the *dph5* mutant grows at approximately the same rate as wild-type cells in rich (YPD) or minimal (SD) medium, we devised a selection scheme based on toxin sensitivity in order to clone the *DPH5* gene. We first placed the toxin F2 gene under *GAL1* control in a yeast integrative plasmid, generating plasmid pLMY109. This plasmid was then integrated at the chromosomal *LEU2* locus of LMY12, yielding strain LMY28 (Fig. 2B), which was used to screen for the presence of the *DPH5* gene. Since the *dph5* mutation is recessive, LMY28 should fail to grow specifically on galactose medium when complemented by the correct gene. In addition, the complementing gene should restore ADP-ribosyl acceptor activity.

Strain LMY28 was transformed with the yeast YCp50

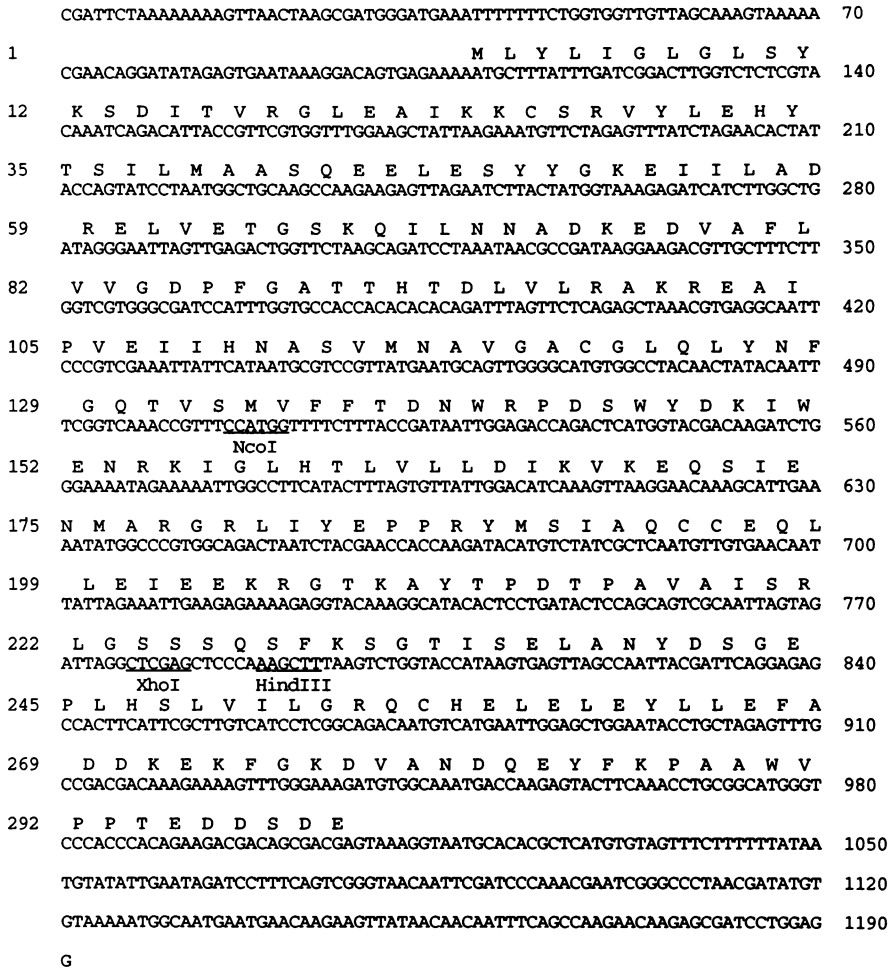


FIG. 5. Nucleotide sequence of the *DPH5* gene and predicted amino acid sequence of its gene product. Nucleotides are numbered on the right; amino acids are numbered on the left. The *Xho*I site used to create the deletion shown in Fig. 4B and the *Nco*I and *Hin*dIII sites used to construct the *dph5* null mutant are shown.

genomic plasmid library (42), and Ura$^+$ transformants were selected on plates containing minimal medium supplemented with glucose. Colonies were then replica plated to minimal plates containing galactose and screened for growth. From approximately 2,500 Ura$^+$ transformants, we found 2 that failed to grow on galactose plates, and both contained significant ADP-ribosyl acceptor activity. For both isolates, segregation of the complementing plasmid resulted in a loss of detectable acceptor activity.

Plasmid DNA from both isolates (designated pLMY111 and pLMY112) yielded similar restriction fragment patterns, and both plasmids complemented the *dph5* mutant strains LMY12 and 0407 but not the *dph2* mutant LMY15. Subclones of pLMY111 in the yeast centromere plasmid pRS316 (46) were tested for complementation of strain LMY12 in vitro by the ADP-ribosyl acceptor assay. A subclone containing a 2.4-kb *Sph*I-*Bam*HI fragment was found to complement the *dph5* mutant fully (Fig. 4A), and complementation by the 2.4-kb *Sph*I-*Bam*HI fragment occurred in both orientations, indicating that the *DPH5* regulatory region was intact.

Sequence analysis of the *DPH5* gene. The 2.4-kb *Sph*I-*Bam*HI fragment containing the *DPH5* gene was subcloned into pBluescript, and nested deletions were created from both ends of the fragment with ExoIII (see Materials and Methods). The nucleotide sequences of both DNA strands were determined. The largest open reading frame was near one end of the fragment and encoded a 300-amino-acid protein with a predicted molecular weight of 33,845. To test whether this open reading frame corresponded to the *DPH5* gene, we subcloned various ExoIII deletions in pRS316 and tested for complementation of LMY12 by the ADP-ribosyl acceptor assay. The sequence of the smallest fragment that still complemented LMY12 extended from the *Sph*I site to nucleotide position 1191 and contained the entire 300-amino-acid open reading frame (Fig. 5). Plasmid pLMY198, carrying this fragment, restored the ADP-ribosyl acceptor activity of strain LMY12 to nearly the same level as the wild-type strain, FY69 (Fig. 4B), indicating this sequence contains the complete *DPH5* gene. Any deletions that extended into the open reading frame coding sequence destroyed acceptor activity, suggesting that this sequence encodes the *DPH5* protein.

We confirmed that pLMY198 contains the *DPH5* gene by demonstrating that the cloned DNA directed integration of a plasmid to the *DPH5* locus. The 1.2-kb *Eco*RI-*Sac*II fragment of pLMY198 was subcloned to the same sites of pRS306 (46), a yeast integrative plasmid, to generate

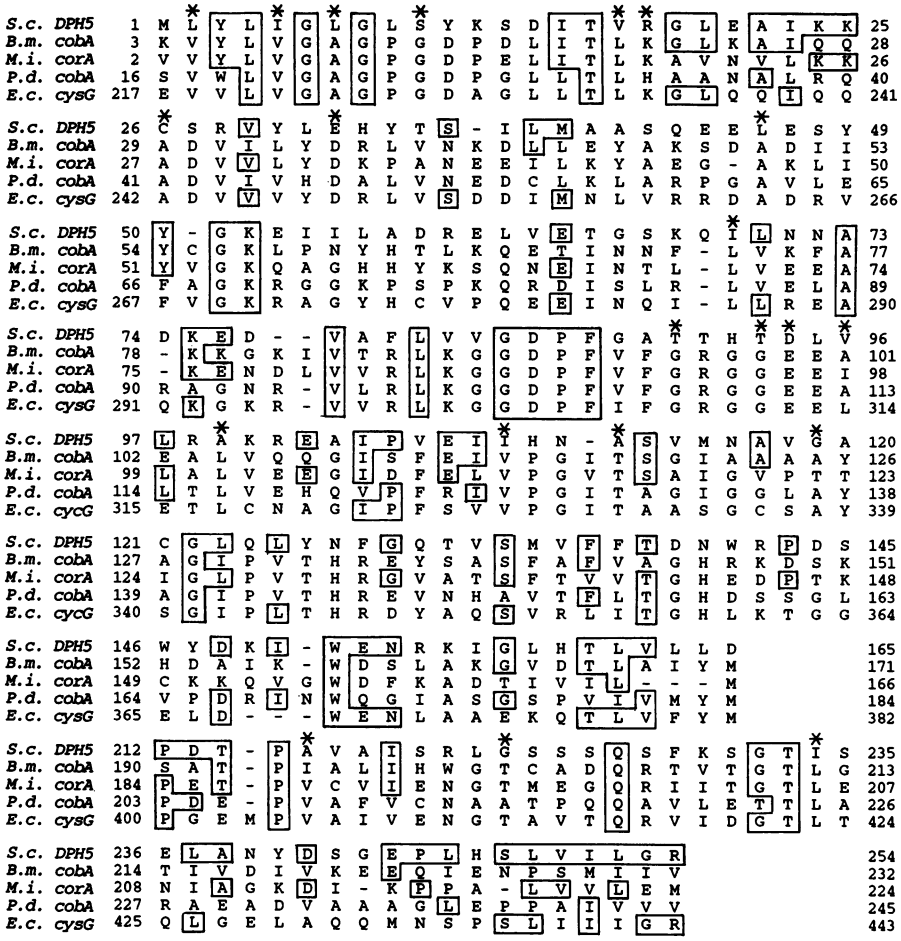


FIG. 6. Alignment of amino acid sequences of the *DPH5* gene product from *S. cerevisiae* (*S.c.*) to SUMT sequences from *B. megaterium* (*B.m.*), *M. ivanovii* (*M.i.*), *P. denitrificans* (*P.d.*), and *E. coli* (*E.c.*) (*cobA*, *corA*, *cobA*, and *cysG* gene products, respectively). Gaps were inserted when necessary for optimal alignment. Open boxes indicate amino acid identity to DPH5, and asterisks refer to positions showing conservative substitutions for all sequences. The numbers on the left and right refer to amino acid position.

pLMY214. Plasmid pLMY214 was linearized at the unique *Bgl*II site within the *DPH5* coding sequence and used to transform strain LMY16 to uracil prototrophy. A Ura^+ transformant (LMY41) was crossed to strain LMY12, and tetrads were dissected. In eight four-spore tetrads, the Dph^+ and Ura^+ phenotypes cosegregated in every tetrad, demonstrating that pLMY214 DNA was tightly linked to the *DPH5* locus.

A data base containing 113 known AdoMet-dependent methyltransferases and other AdoMet-binding proteins was searched for sequences similar to those of the *DPH5* protein with the alignment method FASTA (37). Significant matches were found with AdoMet:uroporphyrinogen III methyltransferases (SUMT) from *Bacillus megaterium* (41), *Methanobacterium ivanovii* (3), *Pseudomonas denitrificans* (10), and *E. coli* (36), enzymes that methylate the C-2 and C-7 of urogen III in the cobalamin (vitamin B_{12}) biosynthetic pathway (3). The similarities to *DPH5* are most prominent within residues 1 to 165 and 212 to 254 (Fig. 6). Amino acid identity within residues 1 to 165 ranges from 21% for *B. megaterium*, *M. ivanovii*, and *E. coli* to 15% for *P. denitrificans*. Conservative substitutions are 25, 30, 25, and 30%, respectively. Residues 1 to 165 include the consensus sequence VXX LXXGDPF (residues 78 to 87 of *DPH5*), which has not been found in other AdoMet-dependent methyltransferases. Region 212 to 254 includes 30 and 25% amino acid identity to sequences from *M. ivanovii* and *E. coli*, respectively, and 16% identity to sequences from *B. megaterium* and *P. denitrificans*. SUMT sequences from *B. megaterium*, *M. ivanovii*, and *P. denitrificans* also have molecular weights (25,800, 24,900, and 29,200, respectively) similar to that predicted for the *DPH5* gene product (33,845). No other sequences with significant similarity to DPH5 were found in the protein sequence data base (National Biomedical Research Foundation Protein Sequence, release 29.0) with the BLAST alignment method (1).

A search of the nucleotide sequence data base (GenBank, release 67.0) for similarities to the entire sequence of the 2.4-kb *Sph*I-*Bam*HI clone, with the BLAST program, revealed a region identical to part of the yeast ubiquitin-ribosomal protein S37 fusion gene *UBI3* (16). The homology covers the last 81 nucleotides of the 2.4-kb clone terminating at the *Bam*HI site (Fig. 4A) and the last 81 nucleotides reported for the 3′ noncoding sequence of *UBI3* (34). The sequence identity suggests that the coding sequences of the *UBI3* and *DPH5* genes are on opposite strands of the DNA, and their respective translation stop codons are separated by 1,851 nucleotides. Further evidence that the *DPH5* gene is

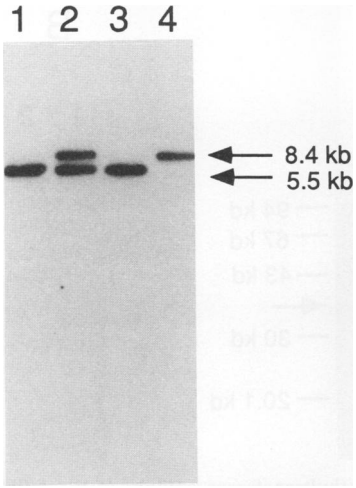


FIG. 7. Southern blot hybridization analysis of *DPH5* gene disruption. Shown is an autoradiogram of *Sal*I-digested genomic DNA after hybridization to a ^{32}P-labelled 1.2-kb *Eco*RI-*Sac*II restriction fragment from pLMY198. Lane 1, Leu$^-$ diploid strain LMY36; lane 2, Leu$^+$ transformant of diploid strain LMY36; lane 3, Leu$^-$ haploid segregant; lane 4, Leu$^+$ haploid segregant. The wild-type *DPH5* gene is contained in a 5.5-kb *Sal*I fragment, and the disrupted gene is contained in an 8.4-kb *Sal*I fragment.

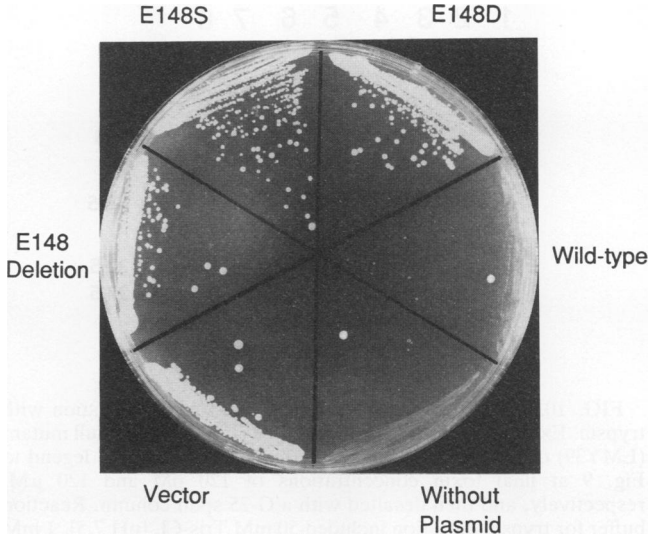


FIG. 8. Growth of the haploid *dph5* null mutant (LMY39) and plasmid-carrying strains of LMY39 on galactose minimal medium. Transformed strains have either plasmid pRS316 or pRS316 derivatives containing the coding sequences for wild-type or Glu-148 mutants of DT fragment A under *GAL1* transcriptional control. The following strains are shown: without plasmid; vector (pRS316); E148 deletion (deletion of Glu-148); E148S (Glu-148 to Ser); E148D (Glu-148 to Asp); and wild-type fragment A. Cells were grown at 30°C for 3 days and photographed.

near the *UBI3* gene is provided by our recent finding that both genes are located on chromosome XII (28, 32).

Disruption of the *DPH5* gene. To determine whether the *DPH5* gene is required for cell viability, we constructed a *dph5* null allele by gene disruption. The *Nco*I-*Hin*dIII restriction fragment internal to *DPH5* was replaced by the *LEU2* gene (Fig. 4; see Materials and Methods), and the resulting *dph5*::*LEU2* allele was integrated into the diploid strain LMY36. Heterozygosity of the resulting strain for the null allele was confirmed by Southern blot analysis (Fig. 7). When this strain (LMY37) was sporulated and the tetrads were dissected, all resulting spores were found to be viable. Haploid strains from eight tetrads were grown, and extracts were prepared and tested for ADP-ribosyl acceptor activity. Segregation of acceptor activity was 2:2, with all toxin-resistant cells being Leu$^+$ and all toxin-sensitive cells being Leu$^-$. No differences in colony size on YPD plates were detected between wild-type and *dph5* null strains. We conclude that the *DPH5* gene is not essential for cell viability.

ADP-ribosylation of EF-2 from the *dph5* null mutant. The *dph5* mutant was selected by virtue of its resistance to the intracellular expression of the F2 fragment of DT, a fragment with reduced ADP-ribosyltransferase activity relative to that of fragment A. To explore the effects of the *DPH5* lesion on toxin sensitivity, we tested the resistance of the *dph5* null mutant to expression of the fully active A fragment of DT and a series of attenuated, mutant forms of fragment A. In other studies, we have constructed a series of *GAL1* promoter expression plasmids that contain the gene for the A fragment of DT and several mutant derivatives of the active-site residue, Glu-148 (17). Two of these mutants, containing changes of Glu-148 to Asp and Glu-148 to Ser, have reductions in NAD:EF-2 ADP-ribosyltransferase activity of 100- and 300-fold, respectively (50), while the third mutant, containing a deletion of Glu-148, has approximately 10^{-6} times the activity of wild-type A fragment (22).

Wild-type yeast transformed with any of these plasmids did not form colonies on minimal plates containing galactose

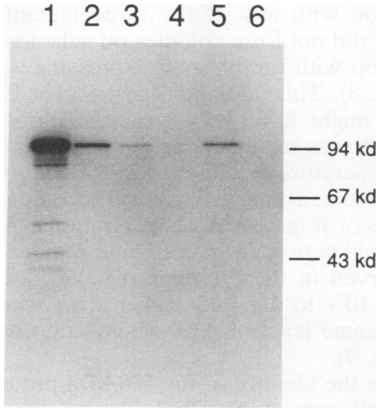


FIG. 9. Autoradiogram showing ADP-ribosyl acceptor activity of extracts from wild-type (lanes 1 to 4) and *dph5* null mutant (lanes 5 and 6) cells. Reactions included 35 μg of total protein from each extract, 63 nM NAD [*adenylate*-^{32}P]NAD (800 Ci/mmol; Amersham), and serial dilutions of DT fragment A in a final volume of 50 μl. After a 30-min incubation at 30°C, reactions were terminated by trichloroacetic acid precipitation, and the pellet was solubilized in SDS sample buffer. Samples were analyzed by SDS–7.5% PAGE followed by autoradiography. Shown are reaction mixtures incubated with the following approximate toxin concentrations: lane 1, 1.2 nM; lane 2, 120 pM; lane 3, 12 pM; lane 5, 120 μM. Samples from reaction mixtures incubated in the absence of toxin are shown in lanes 4 and 6. Densitometric scanning of the autoradiogram showed that the intensity of the 100-kDa EF-2 band from lanes 1 to 3 correlated with toxin concentration. The lower-molecular-weight bands presumably represent proteolytic fragments of EF-2, as described previously (7), and were not further characterized. The locations of molecular mass markers are shown at the right.

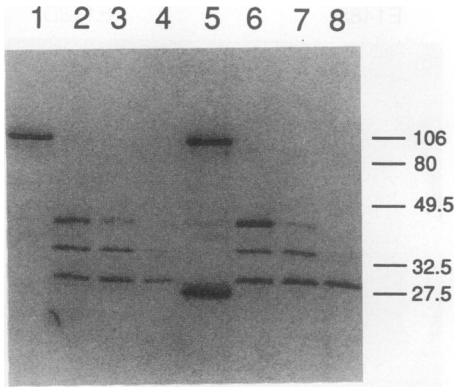


FIG. 10. ADP-ribosylated peptides generated by digestion with trypsin. Extracts prepared from wild-type (FY2) or *dph5* null mutant (LMY39) cells were ADP-ribosylated as described in the legend to Fig. 9 at final toxin concentrations of 120 pM and 120 μM, respectively, and then desalted with a G-25 spun column. Reaction buffer for trypsin digestion included 50 mM Tris-Cl, [pH 7.5], 1 mM EDTA, and 5 mM β-mercaptoethanol in a final volume of 10 μl. Each reaction mixture, containing 1.75 μg of ADP-ribosylated extract and the indicated trypsin concentration, was incubated at 37°C for 30 min. Samples were fractionated by SDS–11.25% PAGE, and the gels were analyzed by scanning on a PhosphorImager (Molecular Dynamics). Shown are the peptide patterns of extracts of FY2 (lanes 1 to 4) and LMY39 (lanes 5 to 8) treated with trypsin at final concentrations of 0 μg/ml (lanes 1 and 5), 10 μg/ml (lanes 2 and 6), 20 μg/ml (lanes 3 and 7), and 50 μg/ml (lanes 4 and 8). The locations of molecular mass markers (in kilodaltons) are shown at the right.

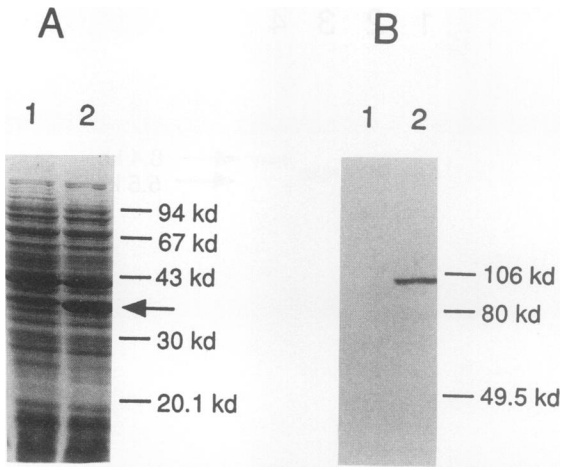


FIG. 11. Methyltransferase activity of the *DPH5* gene product overproduced in *E. coli*. Strain BL21 (DE3) (47) carrying plasmid pT7-7 or pLMY215 was grown at 37°C in 50 ml of LB (2) supplemented with ampicillin (50 μg/ml). At an optical density at 600 nm of 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth continued for 1 h. Cells were harvested by centrifugation, washed, and resuspended in 0.5 ml of sonication buffer (50 mM Tris-Cl [pH 7.5], 2 mM EDTA, 6 mM β-mercaptoethanol, 5 μg of phenylmethylsulfonyl fluoride per ml). Cells were sonicated and centrifuged for 15 min at 15,000 × *g* to isolate the soluble, cytosolic fraction. Equivalent amounts of extract (10 μg of protein) prepared from cells carrying pT7-7 (lanes 1) or pLMY215 (lanes 2) were analyzed for protein by Coomassie staining (A) or methyltransferase activity (B). (A) Coomassie-stained proteins resolved by SDS–11.25% PAGE. Shown on right are molecular mass markers and an arrow indicating the *DPH5* protein. (B) Autoradiogram of methyltransferase reaction products resolved by SDS–7.5% PAGE. Reaction conditions are described in Materials and Methods and included 10 μg of the LMY12 cytosolic extract. Molecular mass markers are shown on right.

(17). The *dph5* null mutant, in contrast, survived after transformation with any of the three mutant fragment A plasmids but did not form colonies on galactose plates after transformation with the plasmid expressing wild-type fragment A (Fig. 8). This finding suggested that EF-2 from the null mutant might have very weak ADP-ribosyl acceptor activity which was undetectable under standard assay conditions. We therefore tested extracts of wild-type cells and the *dph5* null mutant for ADP-ribosyl acceptor activity over a wide range of fragment A concentrations. ADP-ribosylation of a 100-kDa protein in *dph5* null mutant extracts was indeed observed in the presence of concentrations of DT fragment A 10^6- to 10^7-fold higher than were needed to achieve the same level of ADP-ribosylation with wild-type extracts (Fig. 9).

To confirm the identity of the 100-kDa protein present in the *dph5* null extract as EF-2, we analyzed the ADP-ribosylated proteins in wild-type and mutant extracts by peptide mapping. Following ADP-ribosylation, extracts were treated with various concentrations of trypsin, and the radiolabelled cleavage products were analyzed by SDS-PAGE. The peptide patterns of wild-type and *dph5* mutant extracts were similar and included three bands with apparent molecular masses of 44, 36, and 30 kDa (Fig. 10). We also found that the peptide pattern after digestion with chymotrypsin was similar for wild-type and mutant extracts. These results confirm that EF-2 from the *dph5* mutant is ADP-ribosylated and strongly suggest that ribosylation of the mutant occurs at the same modified histidine residue as does that of wild-type EF-2.

Functional expression of the *DPH5* protein. To probe the possibility that the *DPH5* protein product participates directly in methylation of EF-2, we expressed the *DPH5* gene in *E. coli*, an organism lacking the diphthamide biosynthetic pathway. A plasmid (pLMY215) containing the complete *DPH5* coding sequence was constructed under the transcriptional control of the bacteriophage T7 promoter (see Materials and Methods), and the soluble cytosolic fraction of induced cultures carrying pLMY215 or the vector control pT7-7 was analyzed for protein and EF-2 methyltransferase activity. The extract from cells expressing *DPH5* showed overproduction of a protein with a molecular mass (34.5 kDa) similar to the predicted value for DPH5 (Fig. 11A, lane 2). This extract was active in methylation of EF-2 when tested in combination with the *dph5* mutant extract in the methyltransferase assay (Fig. 11B, lane 2). We also partially purified EF-2 from the *dph5* null mutant LMY39 and found that both methyl acceptor and weak ADP-ribosyl acceptor activities coeluted in a single chromatographic peak (Fig. 12). These results imply that *DPH5* directly participates in methylation of EF-2, although the possibility that an *E. coli* protein is also required for activity cannot be ruled out.

DISCUSSION

Diphthamide is the most complex posttranslationally modified amino acid known to date (13). As a first attempt to understand the genetics of diphthamide biosynthesis and its possible role in protein synthesis, we have isolated a diphthamide mutation of *S. cerevisiae*, *dph5*, and characterized the *DPH5* gene and its protein product. The data presented

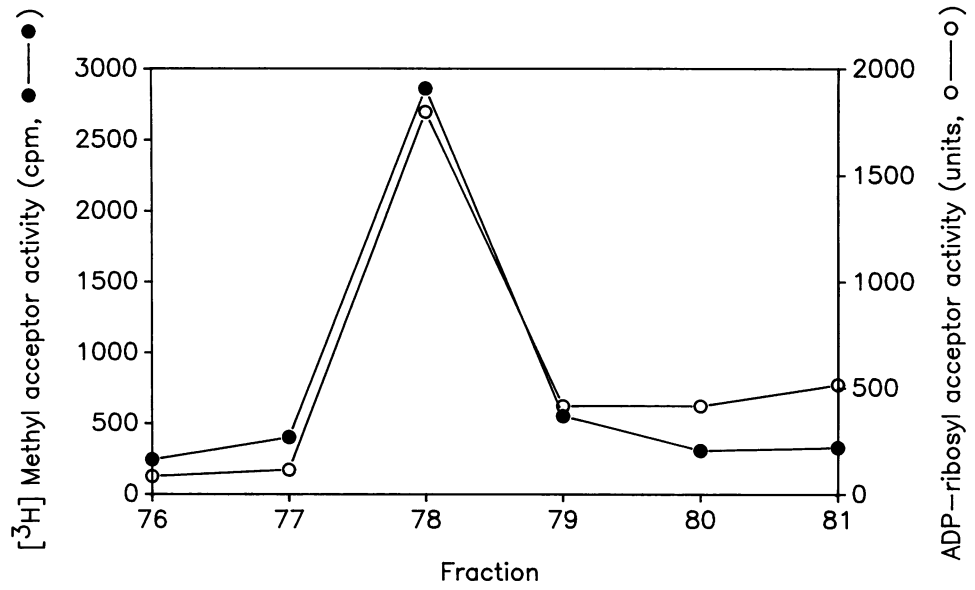


FIG. 12. Methyl acceptor and ADP-ribosyl acceptor activities of EF-2 partially purified from the *dph5* null mutant strain LMY39. EF-2 purification and assay are described in Materials and Methods. Shown are Mono Q column fractions assayed separately for *E. coli*-produced *DPH5* methyl acceptor activity (closed circles) and DT fragment A ADP-ribosyl acceptor activity (open circles).

demonstrate that the 300-amino-acid *DPH5* gene product is involved in the AdoMet-dependent methyltransferase step of diphthamide biosynthesis. We showed that EF-2 from the mutant served as an acceptor for label from methyl-labelled AdoMet but not from carboxyl-labelled AdoMet in extracts complemented with extracts from wild-type yeast or the *dph2* mutant. Furthermore, the amino acid sequence of DPH5 exhibited significant similarity to the sequences of AdoMet-dependent methyltransferases (SUMT) from various bacterial species. Finally, we showed that *DPH5* overproduced in *E. coli* methylates EF-2 partially purified from the *dph5* mutant.

DPH5 and SUMT may represent a distinct family of AdoMet-dependent methyltransferases, unrelated or distantly related to other methyltransferases. The homology between DPH5 and SUMT is greater than that found among other AdoMet-dependent methyltransferases, and the sequence motifs common among other methyltransferases, including those that act on DNA, RNA, or small molecules, are absent or very weak in DPH5 and SUMT (20, 40). The sequence similarities between DPH5 and SUMT are also interesting in view of their functional differences (SUMT methylates a carbon versus a nitrogen atom for DPH5) and evolutionary differences (prokaryotic versus eukaryotic origin). Our development of an *E. coli* expression system for *DPH5* should facilitate further studies on the functional role of these conserved residues by site-directed mutagenesis.

One goal of this work was to construct and characterize a yeast strain in which a chromosomal gene involved in diphthamide biosynthesis was disrupted. Diphthamide mutants that had been isolated earlier were selected solely on the basis of toxin resistance, and it was conceivable that there had been inadvertent selection for leaky mutants that remained viable. We therefore created a null mutation of the chromosomal *DPH5* gene, in which the middle third of the *DPH5* coding sequence was replaced with a 3.1-kb *LEU2* fragment, and tested the resulting mutant for viability and loss of ADP-ribosyl acceptor activity. The results imply that *DPH5* and diphthamide are not essential for cell viability, in agreement with results of earlier studies with toxin-resistant mammalian and yeast cells (8, 23, 30).

While intact diphthamide is not necessary for normal protein synthesis, previous studies imply that the precursor histidine is required (33). Omura and coworkers (33) used site-directed mutagenesis to show that substitution of aspartate, arginine, or lysine for the precursor histidine residue 715 in hamster EF-2 results in a loss of function of the factor in vivo. Whether the histidine must be modified in any way to function in protein synthesis remains unknown.

Although the *dph5* mutant was originally selected by virtue of its resistance to expression of the F2 fragment of DT, the mutant proved, surprisingly, to be sensitive to the expression of wild-type fragment A. The *dph5* null mutant also showed a similar difference in sensitivity to expressed F2 and fragment A. These results suggested that EF-2 from the *dph5* mutants might in fact show very weak ADP-ribosyl acceptor activity. F2, which is less active enzymically than fragment A, had thus been a fortuitous choice for our selection of mutants. The magnitude of the enzymic deficit of F2 is difficult to determine, since measurement of its activity is highly sensitive to proteolytic attack and reduction at the junction between fragments A and B. However, we found that expression of fragment A containing a Glu-148 to Asp mutation, a Glu-148 to Ser mutation, or a deletion of Glu-148 (100-, 300-, and 10^6-fold less active, respectively, than wild-type fragment A) killed wild-type yeast but not the *dph5* null mutant.

Subsequently we showed that EF-2 from the *dph5* null mutant did indeed have weak activity as a substrate for ADP-ribosylation, between 10^{-6} and 10^{-7} times that of wild-type EF-2. Our in vitro studies imply that the structure of the diphthamide precursor in the *dph5* mutant includes the four-carbon backbone from AdoMet (precursor 2) but is missing at least one of the three donated methyl groups found in the final diphthamide structure 4. It is generally regarded that precursor 2 but not diphthine 3 can confer

complete toxin resistance in vivo and in vitro (7, 31). Our results imply, however, that complete methylation of precursor 2 is not absolutely essential for ADP-ribosyl acceptor activity. Since previous studies found only a 33-fold decrease in the ADP-ribosyl acceptor activity of yeast EF-2 containing diphthine 3 compared with that of yeast EF-2 containing diphthamide 4 (7), the trimethylated amine may play a significant role in ADP-ribosyl acceptor activity.

No physiological function for diphthamide has yet been determined. It has been suggested that the residue may serve as the target for an endogenous cellular NAD:EF-2 ADP-ribosyltransferase (26) and that this reaction may serve some function in regulating protein synthesis. Weak activity in ADP-ribosylating EF-2 has been detected in a variety of eukaryotic cells, including polyomavirus-transformed baby hamster kidney cells growing under a low serum concentration (15). If diphthamide does indeed serve as a site of physiological ADP-ribosylation, our data would suggest that the rate of the reaction in the *dph5* null mutant may be greatly diminished but not abolished, since toxin-dependent acceptor activity is still detectable.

Our approach for selecting diphthamide mutants in yeast cells, which relies on the intracellular expression of toxin (38), avoids the technical difficulties inherent in working with yeast spheroplasts (8) and the potential selection of host mutations affecting toxin entry. By expressing DT fragment A derivatives that are attenuated to various degrees, it should be possible to control the level of ADP-ribosyltransferase activity within cells over many orders of magnitude and hence to isolate diphthamide mutants differing in toxin resistance. Further biochemical and physiological analysis of such mutants may reveal a functional role for this unique biosynthetic pathway.

ACKNOWLEDGMENTS

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Blanche, F., C. Robin, M. Couder, D. Faucher, L. Cauchois, B. Cameron, and J. Crouzet.** 1991. Purification, characterization, and molecular cloning of *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase from *Methanobacterium ivanovii*. J. Bacteriol. **173:**4637–4645.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41:**458–472.
5. **Carroll, S. F., J. T. Barbieri, and R. J. Collier.** 1988. Diphtheria toxin: purification and properties. Methods Enzymol. **165:**68–76.
6. **Carroll, S. F., and R. J. Collier.** 1988. Diphtheria toxin: quantification and assay. Methods Enzymol. **165:**218–225.
7. **Chen, J.-Y. C., and J. W. Bodley.** 1988. Biosynthesis of diphthamide in *Saccharomyces cerevisiae*. Partial purification and characterization of a specific *S*-adenosylmethionine:elongation factor 2 methyltransferase. J. Biol. Chem. **263:**11692–11696.
8. **Chen, J.-Y. C., J. W. Bodley, and D. M. Livingston.** 1985. Diphtheria toxin-resistant mutants of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5:**3357–3360.
9. **Collier, R. J., and J. J. Mekalanos.** 1980. ADP-ribosylating exotoxins, p. 261–291. *In* H. Bisswanger and E. Schmincke-Ott (ed.), Multifunctional proteins. John Wiley & Sons, Inc., New York.
10. **Crouzet, J., L. Cauchois, F. Blanche, L. Debussche, D. Thibaut, M.-C. Rouyez, S. Rigault, J.-F. Mayaux, and B. Cameron.** 1990. Nucleotide sequence of *Pseudomonas denitrificans* 5.4-kilobase DNA fragment containing five *cob* genes and identification of structural genes encoding *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase and cobyrinic acid *a,c*-diamide synthase. J. Bacteriol. **172:**5968–5979.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
12. **Dunlop, P. C., and J. W. Bodley.** 1983. Biosynthetic labeling of diphthamide in *Saccharomyces cerevisiae*. J. Biol. Chem. **258:**4754–4758.
13. **Evans, D. A., and K. M. Lundy.** 1992. Synthesis of diphthamide: the target of diphtheria toxin catalyzed ADP-ribosylation in protein synthesis elongation factor 2. J. Am. Chem. Soc. **114:**1495–1496.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
15. **Fendrick, J. L., and W. J. Iglewski.** 1989. Endogenous ADP-ribosylation of elongation factor 2 in polyoma virus-transformed baby hamster kidney cells. Proc. Natl. Acad. Sci. USA **86:**554–557.
16. **Finley, D., B. Bartel, and A. Varshavsky.** 1989. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature (London) **338:**394–401.
17. **Fu, H., L. C. Mattheakis, and R. J. Collier.** Unpublished data.
18. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagaloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
19. **Hoffman, C., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
20. **Ingrosso, D., A. V. Fowler, J. Bleibaum, and S. Clarke.** 1989. Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. Common sequence motifs for protein, DNA, RNA, and small molecule *S*-adenosylmethionine-dependent methyltransferases. J. Biol. Chem. **264:**20131–20139.
21. **Ito, H., K. Fukuda, K. Marata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
22. **Kochi, S., and R. J. Collier.** Unpublished data.
23. **Kohno, K., T. Uchida, E. Mekada, and Y. Okada.** 1985. Characterization of diphtheria-toxin-resistant mutants lacking receptor function or containing nonribosylatable elongation factor 2. Somatic Cell Mol. Genet. **11:**421–431.
24. **Kohno, K., T. Uchida, H. Ohkubo, S. Nakanishi, T. Nakanishi, T. Fukui, E. Ohtsuka, M. Ikehara, and Y. Okada.** 1986. Amino acid sequence of mammalian elongation factor 2 deduced from the cDNA sequence: homology with GTP-binding proteins. Proc. Natl. Acad. Sci. USA **83:**4978–4982.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
26. **Lee, H., and W. J. Iglewski.** 1984. Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and pseudomonas toxin A. Proc. Natl. Acad. Sci. USA **81:**2703–2707.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.
28. **Mattheakis, L. C., F. Sor., and R. J. Collier.** Unpublished data.
29. **Moehring, J. M., and T. J. Moehring.** 1988. The post-translational trimethylation of diphthamide studied in vitro. J. Biol. Chem. **263**:3840–3844.
30. **Moehring, J. M., T. J. Moehring, and D. E. Danley.** 1980. Posttranslational modification of elongation factor 2 in diphtheria-toxin-resistant mutants of CHO-K1 cells. Proc. Natl. Acad. Sci. USA **77**:1010–1014.
31. **Moehring, T. J., D. E. Danley, and J. M. Moehring.** 1984. In vitro biosynthesis of diphthamide studied with mutant Chinese hamster ovary cells resistant to diphtheria toxin. Mol. Cell. Biol. **4**:642–650.
32. **Monia, B. P., K. M. Haskell, J. R. Ecker, D. J. Ecker, and S. T. Crooke.** 1989. Chromosomal mapping of the ubiquitin gene family in *Saccharomyces cerevisiae* by pulsed field gel electrophoresis. Nucleic Acids Res. **17**:3611–3612.
33. **Omura, F., K. Kohno, and T. Uchida.** 1989. The histidine residue of codon 715 is essential for function of elongation factor 2. Eur. J. Biochem. **180**:1–8.
34. **Ozkaynak, E., D. Finley, M. J. Solomon, and A. Varshavsky.** 1987. The yeast ubiquitin genes: a family of natural gene fusions. EMBO J. **6**:1429–1439.
35. **Pappenheimer, A. M., Jr., P. C. Dunlop, K. W. Adolph, and J. W. Bodley.** 1983. Occurrence of diphthamide in archaebacteria. J. Bacteriol. **153**:1342–1347.
36. **Peakman, T., J. Crouzet, J. F. Mayaux, S. Busby, S. Mohan, N. Harborne, J. Wootton, R. Nicolson, and J. Cole.** 1990. Nucleotide sequence, organisation and structural analysis of the products of genes in the *nirB-cysG* region of the *Escherichia coli* K-12 chromosome. Eur. J. Biochem. **191**:315–323.
37. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA **85**: 2444–2448.
38. **Perentesis, J. P., F. S. Genbauffe, S. A. Veldman, C. L. Galeotti, D. M. Livingston, J. W. Bodley, and J. R. Murphy.** 1988. Expression of diphtheria toxin fragment A and hormone-toxin fusion proteins in toxin-resistant yeast mutants. Proc. Natl. Acad. Sci. USA **85**:8386–8390.
39. **Pfeifer, K., B. Arcangioli, and L. Guarente.** 1987. Yeast *HAP1* activator competes with the factor rc2 for binding to the upstream activation site UAS1 of the *CYC1* gene. Cell **49**:9–18.
40. **Posfai, J., A. S. Bhagwat, G. Posfai, and R. J. Roberts.** 1989. Predictive motifs derived from cytosine methyltransferases. Nucleic Acids Res. **17**:2421–2435.
41. **Robin, C., F. Blanche, L. Cauchois, B. Cameron, M. Couder, and J. Crouzet.** 1991. Primary structure, expression in *Escherichia coli*, and properties of *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase from *Bacillus megaterium*. J. Bacteriol. **173**:4893–4896.
42. **Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink.** 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene **60**:237–243.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
44. **Sayers, J. R., W. Schmidt, and F. Eckstein.** 1988. 5′-3′ exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. Nucleic Acids Res. **16**:791–802.
45. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**:19–27.
47. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
48. **Tweten, R. K., and R. J. Collier.** 1983. Molecular cloning and expression of gene fragments from corynebacteriophage β encoding enzymatically active peptides of diphtheria toxin. J. Bacteriol. **156**:680–685.
49. **Van Ness, B. G., J. B. Howard, and J. W. Bodley.** 1980. ADP-ribosylation of elongation factor 2 by diphtheria toxin: isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. J. Biol. Chem. **255**:10717–10720.
50. **Wilson, B. A., K. A. Reich, B. R. Weinstein, and R. J. Collier.** 1990. Active-site mutations of diphtheria toxin: effects of replacing glutamic acid-148 with aspartic acid, glutamine, or serine. Biochemistry **29**:8643–8651.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
52. **Yocum, R. R., S. Hanley, R. West, Jr., and M. Ptashne.** 1984. Use of *lacZ* fusions to delimit regulatory elements of the inducible divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1985–1998.