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Cloning and Characterization of *Saccharomyces cerevisiae* Genes That Confer L-Methionine Sulfoximine and Tabtoxin Resistance

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Pseudomonas tabaci **produces a toxin, tabtoxin, that causes wildfire disease in tobacco. The primary target of tabtoxin is presumed to be glutamine synthetase. Some effects of tabtoxin in tobacco can be mimicked by methionine sulfoximine (MSO), a compound that is known to inactivate glutamine synthetase. To understand how organisms can be made resistant to tabtoxin and MSO, we used *Saccharomyces cerevisiae*. We demonstrate that yeast strains carrying the glutamine synthetase gene, *GLN1*, on a multicopy plasmid overproduced glutamine synthetase and showed increased drug resistance. These and other data indicate that glutamine synthetase is the primary target of tabtoxin and MSO in *S. cerevisiae*. We also isolated three *S. cerevisiae* DNA inserts of 2.1, 2.3, and 2.8 kilobases that conferred tabtoxin and MSO resistance when the inserts were present on a multicopy plasmid. These plasmids conferred resistance to MSO by blocking intracellular transport of the drug. Transport appeared to occur by one or more methionine permeases. Resistance to tabtoxin could also occur by blockage of intracellular transport, but the drug was transported by some permease other than a methionine permease. These drug resistance plasmids did not block transport of citrulline, indicating that they did not affect the general amino acid permease.**

Wildfire is an infectious leafspot disease of tobacco. The chlorotic leaf symptom that characterizes the disease is caused by tabtoxin (32), a phytotoxic dipeptide produced by *Pseudomonas tabaci*. Tabtoxin is a novel β-lactam-containing amino acid, 2-amino-4-(3-hydroxy-2-oxoazacyclobutan-3-yl)butanoic acid, linked by a peptide bond to the amino group of either serine or threonine (32). Tabtoxin inhibits glutamine synthetase activity in tobacco in vivo, causing accumulation of ammonium (8, 30, 34). Inhibition of glutamine synthetase has at least two effects, either one of which could cause cell death. First, the enzyme is probably the only major route for glutamine synthesis in plants, and second, the glutamine synthetase-glutamate synthase pathway is the only efficient way to detoxify the ammonia released by nitrate reduction, amino acid degradation, or photorespiration in most plants (17).

Inhibition of glutamine synthetase by tabtoxin can be prevented by glutamine (30), which suggests that glutamine synthetase is the only cellular target in tobacco. Methionine sulfoximine (MSO), known to be a potent and highly specific inhibitor of glutamine synthetase (22), causes chlorotic lesions in tobacco leaves, and these effects are also prevented by glutamine (30). Because it appears to mimic the effects of tabtoxin and because it is commercially available, whereas tabtoxin is not, MSO has been used as a tool for studying wildfire disease in tobacco. Carlson (3) isolated MSO-resistant tobacco cells from callus cultures and showed that plants derived from them were resistant to the chlorotic affects of both MSO and *P. tabaci*. In some lines the MSO-resistant trait behaved as a single dominant locus. The molecular mechanism underlying the resistance trait remains unknown. *Nicotiana longiflora* is a species of tobacco that is naturally resistant to wildfire. It has been possible to transfer resistance to burley tobacco, but not to flue-cured commercial cultivars of tobacco, by standard breeding techniques (20). The wildfire resistance trait behaves as a single dominant locus. The molecular mechanism underlying this trait is not known, nor is it known whether this natural resistance trait is related to the MSO resistance trait described by Carlson.

As part of a long-term study to understand how tabtoxin and MSO kill tobacco cells and how the *N. longiflora* resistance gene functions, we undertook studies of tabtoxin and MSO resistance in *Saccharomyces cerevisiae*. The rationale for our experiments is based on the observations of Rine et al. (27) that *S. cerevisiae* can be made resistant to metabolic inhibitors by increasing the concentration of the enzyme or protein that is the target of the inhibitor. Increased protein is obtained by molecularly cloning the gene that codes for the protein on a multicopy *S. cerevisiae* recombinant DNA vector. This in effect is an example of cellular resistance due to gene amplification. We reasoned that *S. cerevisiae* could be made resistant to tabtoxin and MSO by putting its glutamine synthetase gene, *GLN1* (24), on a multicopy vector. Our results confirmed this prediction. We also believed that there might be other yeast genes that would confer resistance and that these would be easy to select from a yeast gene bank. This approach enabled us to select yeast genes that confer MSO and tabtoxin resistance on yeast when present on a multicopy vector. These genes function by preventing uptake of MSO.

MATERIALS AND METHODS

Bacterial and yeast strains. *Escherichia coli* DG75 (F⁻ *hsdS1 leu-6 ara-14 galK2 xyl-5 mtl-1 rpsL20 thi-1 supE44* Δ*lacZ39* λ⁻) and HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻) were used as hosts for transformation and propagation of plasmids. *S. cerevisiae* strains used were DC5 (**a** *leu2-3, leu2-112, his-3 can1-11*), DBY745 (α *ura3-52, leu2-3, leu2-112, ade1-100*), 745-gln1 (α *ura3-52, leu2-3, leu2-112, ade1-100, gln1-754* [24]), SJ21 (**a** *gal4-2, ura3-52, leu2-3, leu2-112, ade1, MEL1* [15]), and D13-1A (**a** *his3-532 trp1-289 gal2* [33]).

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Media. YPD medium contained 20 g of glucose, 20 g of peptone, and 10 g of yeast extract per liter. Synthetic medium contained (per liter) 3.4 g of yeast nitrogen base (Difco Laboratories) without amino acids or ammonium sulfate, 20 g of glucose, auxotrophic supplements at 20 μg/ml, and as nitrogen sources, either 2.0 g of glutamine (GGln), 1.25 g of potassium glutamate (GGlt), 2.0 g of ammonium sulfate (GN), 2.0 g of ammonium sulfate and 1.25 g of potassium glutamate (GNGlt), or 1.0 g of proline (GProl). YPDgln contained 3.0 g of glutamine per liter.

Preparation of yeast extracts. Log-phase cells were washed and suspended in breaking buffer (5 mM morpholineethanesulfonic acid [MES], 5 mM $MnCl_2$, pH 6.3, and 1 mM phenylmethylsulfonyl fluoride or 0.1 M Tris, 0.2 mM ADP, pH 7.0, and 1 mM phenylmethylsulfonyl fluoride) at about 100 A_{600} units per ml. Cells were disrupted by vortexing with 1/2 volume of 0.5-mm glass beads for six 30-s periods at 4°C. After vortexing, the liquid phase was removed to a 1.5-ml microfuge tube and centrifuged for 5 min at 4°C. The supernatant fluid was diluted and used immediately for measuring enzyme activity, or it was stored undiluted for up to 2 months at −80°C without loss of enzyme activity.

Enzyme activities. The transferase activity of glutamine synthetase was measured as described by Mitchell and Magasanik (25). One unit of transferase activity catalyzes the formation of 1 μM of γ-glutamylhydroxamate per min at 30°C. NAD-dependent glutamate dehydrogenase activity was measured as an α-ketoglutarate- and ammonium ion-dependent oxidation of NADH (5). ADP (0.2 mM) was included in the reaction mixture to stabilize the enzyme. The decrease in absorbance at 340 nm was measured simultaneously in the test sample and in the control which lacked α-ketoglutarate. One unit of enzyme activity represents 1 nM of NADH oxidized per min per mg of protein at 25°C.

Permease activity. Early-log-phase cells, grown in GNGlt medium at 30°C, were collected by filtration and washed with and suspended in minimal medium (3.4 g of yeast nitrogen base without amino acids and ammonium sulfate and 20 g of glucose per liter with auxotrophic supplements) prewarmed to 30°C, at a density of 1 to 2 A_{600} units per ml. The cells were aerated for 2 min at 30°C. A sample was removed to measure the protein concentration. The radioactive compound was added, and samples of the culture were rapidly filtered (0.4-μm pore size; Nuclepore Corp., Pleasanton, Calif.) at 30-s intervals for 2 min. Filters were washed with ice-cold minimal medium containing a 20-fold excess of the unlabeled compound and dried, and the radioactivity was counted to determine the initial rate of transport. Units of permease activity are expressed as nanomoles of compound accumulated per minute. [^{35}S]methionine (400 Ci/mmol; New England Nuclear Corp.), [^{35}S]MSO (2.3×10^6 cpm/μmol), and [^{14}C]citrulline (40 mCi/mmol; Amersham Corp.) were used to measure methionine, MSO, and the general amino acid permease (GAP), respectively.

Yeast transformation. *S. cerevisiae* was transformed by the method of Hinnen et al. (13). Strain 745-gln1 was transformed by the procedure of Ito et al. (14).

Plasmid copy number. Total DNA was isolated (33) from exponential-phase yeast cultures grown in GNGlt medium supplemented with leucine for YEp24 transformants or uracil for YEp13 transformants. Further purification was obtained by extraction with phenol and then chloroform. The ratio of plasmid DNA to genomic DNA was determined by the DNA dot-blotting procedure of Gillespie and Bresser (10). A dot of 2 ng of plasmid pTCM DNA (9) was made on each nitrocellulose filter so that filters hybridized to different probes could be compared. Plasmid pTCM carries pBR322 sequences which are shared with pMSO plasmids, and it also carries the gene coding for the ribosomal L protein (9). Two identical blots were made. One was hybridized to the ^{32}P-oligolabeled *Bam*HI-*Ava*I fragment from the ribosomal L protein coding region. This blot was used to normalize the amount of total yeast DNA per blot, since the L gene is present in one copy per cell (9). The other blot was hybridized to ^{32}P-oligolabeled pBR322, which would detect pMSO sequences. Radioactivity in each dot was determined by scintillation counting. All transformants contained 3 to 6 copies of the plasmid, with the exception of SJ21(YEp13) and SJ21(pMSO1), which contained 9 and 11 copies, respectively. In all transformed strains at least 90% of the cells contained a plasmid, as measured by plating samples on YPD plates and minimal uracil or leucine selection plates.

Glutamine synthetase mRNA. To measure the relative amount of glutamine synthetase mRNA, total yeast RNA was isolated by the method of Carlson and Botstein (2) and dot-blotted to duplicate nitrocellulose filters under conditions for preferentially binding polyadenylated [poly(A)$^+$] RNA (10). To normalize the amount of mRNA bound for each sample, one filter was hybridized with the same ^{32}P-oligolabeled L protein probe that was used for the plasmid copy number determinations. The other blot was hybridized to a ^{32}P-oligolabeled 0.8-kilobase (kb) *Eco*RI DNA fragment from *GLN1*. Autoradiograms were quantitated by densitometry, and the data were normalized to the glutamine synthetase mRNA level of strain SJ21.

Purification of tabtoxin. Tabtoxin and tabtoxinine were isolated from culture filtrates of *Pseudomonas syringae* pv. *tabaci* CHS-2 by the procedure of Stewart (32), except that the first ion-exchange column was equilibrated with distilled water acidified to pH 2.5 with HCl instead of 1 M NaCl and the second ion-exchange column was eluted with 0.2 M ammonium formate, pH 3.4, instead of pyridine acetate. Tabtoxinine eluted at 1.5 to 2 column volumes and tabtoxin at 4 to 5 column volumes. Tabtoxin and tabtoxinine were located by analysis with ninhydrin, by assay with an amino acid analyzer, or by a tobacco leaf bioassay (18). In an improved and more rapid assay we used a Bio-Rad Aminex A9 cation-exchange high-pressure liquid chromatography column equilibrated and eluted in 0.2 M NaH_2PO_4, pH 3.3, prepared by adjusting the pH with NaOH. The column was monitored at 205 nm with a Schoeffel Spectraflow UV-vis monitor, and tabtoxin was located in the eluate by comparison with purified tabtoxin. A typical preparation from 5 liters of culture filtrate containing 75 mg of tabtoxin yielded 56 mg of tabtoxin from the second ion-exchange column (0.82 by 90 cm) along with 2.5 mg of tabtoxinine.

Miscellaneous procedures. L-[^{35}S]MSO was synthesized from L-[^{35}S]methionine (400 Ci/mmol) by the procedure of Bentley et al. (1). Small- and large-scale preparations of plasmid DNAs were obtained from *E. coli* by the alkaline lysis method described by Maniatis et al. (21), and plasmid DNA was purified by CsCl-ethidium bromide density gradient centrifugation. Southern blotting and hybridization procedures have been described (31). Protein concentration was measured by the procedure of Lowry et al. (19). DNA probes were oligolabeled with [^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by the method of Feinberg and Vogelstein (7). For selecting MSO-resistant transformants, cells were first plated in 8 ml of GGlt regeneration agar (13) on a 25-ml GGlt base plate. The layer of regeneration agar was overlaid with another 8 ml of GGlt regeneration agar, and after 6 h of incubation at 30°C, MSO was spread onto the

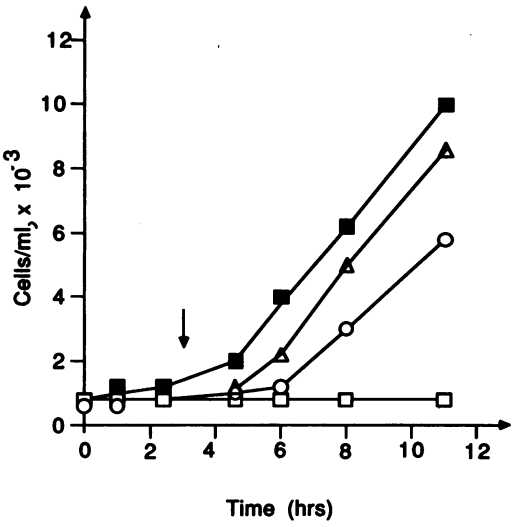

FIG. 1. L-Methionine and L-glutamine reverse the growth-inhibiting affect of MSO. A culture of SJ21(YEp13) was grown to the late exponential phase in GNGlt medium and then diluted to 500 cells per ml in the same medium with or without 1 mM MSO. After 3 h of growth at 30°C, 14 mM glutamine or 1 mM methionine was added to the cultures containing MSO at the time indicated by the arrow. At various times samples were removed, diluted, and plated on YPD plates to calculate the number of colony-forming cells. Symbols: ■, no MSO; □, 1 mM MSO; △, 1 mM MSO plus glutamine; ○, 1 mM MSO plus methionine.

plate (41 ml total volume) to give a final concentration of 1 mM MSO.

RESULTS

Effect of L-MSO and tabtoxin on growth of *S. cerevisiae*. A preliminary estimation of the minimum concentration of MSO that inhibits growth of *S. cerevisiae* was made by spreading 10^2 to 10^3 cells, pregrown to saturation in YPD medium, onto GN plates containing various concentrations of MSO. The concentration of MSO that prevented colony formation was specific for each strain. The most sensitive strains, DBY745 and D13-1A, failed to grow on 1 mM MSO in the presence of 1% ammonium sulfate. The most resistant strain, DC5, did not grow on 20 mM MSO when 1% ammonium sulfate was used as a nitrogen source. When the concentration of ammonium sulfate was lowered to 0.1%, the strain became more sensitive to MSO, and growth was inhibited by 2 mM MSO. Growth inhibition was also analyzed by growing cells in liquid GN medium containing different concentrations of MSO. In all strains, the growth rate was proportional to the concentration of MSO, up to a drug concentration that completely inhibited growth (data not shown). The inhibitory concentration correlated with the data obtained on plates.

If cell growth was inhibited due to inactivation of glutamine synthetase activity by MSO, then glutamine should relieve growth inhibition. We found that 14 mM glutamine rapidly reversed the growth-inhibiting effect of MSO (Fig. 1). Other concentrations of glutamine were not examined.

Yeast might also be spared from killing by MSO if the drug were excluded from the cell. It has been reported that *Chlorella vulgaris* transports MSO by an energy-dependent, membrane-bound carrier. Methionine can prevent the toxic effect of MSO, presumably by competing for this membrane carrier and blocking MSO uptake (23). To determine whether MSO and methionine use the same membrane transporter(s) in *S. cerevisiae*, we examined the toxic effect of MSO in the presence and absence of methionine. L-Methionine relieved MSO inhibition when added to the medium at the same concentration as MSO (Fig. 1). As a control, we studied growth inhibition by MSO in medium containing a mixture of amino acids (except L-glutamine and L-methionine) that are not transported by the methionine transporter and that are not structurally similar to MSO. Full growth inhibition was observed in all media, showing that methionine specifically overcomes the inhibitory effect of MSO. More evidence that MSO is transported by a methionine transport system is given below.

The growth-inhibiting effect of tabtoxin on strain SJ21 was studied. However, the experiments could not be conducted in the same way as for MSO because tabtoxin is not commercially available and we had limited amounts. Typically, *S. cerevisiae* were inoculated at 1,000 cells per ml in 1 ml of GNGlt medium, and growth of the culture was monitored by counting colonies plated on YPD plates. In this type of experiment we found that 1 mM tabtoxin inhibited growth by 60 to 80%. As with MSO, growth inhibition did not occur when the medium was supplemented with 14 mM L-glutamine, suggesting that tabtoxin was inhibiting glutamine synthetase. Addition of 1 mM L-methionine to the medium did not restore normal growth of the culture, suggesting that tabtoxin does not use the methionine transporter.

In vitro effects of MSO and tabtoxin on glutamine synthetase activity in cell extracts. The results of the preceding section predicted that MSO and tabtoxin should inhibit glutamine synthetase activity in yeast. The inhibitory effect of these two compounds was studied in cell extracts of *S. cerevisiae* by measuring the transferase activity of glutamine synthetase. Inhibition of enzyme activity by MSO was very rapid and was complete after 10 min of preincubation (Fig. 2). The inhibitory effect of tabtoxin increased with preincubation time (Fig. 2). Maximal inhibition of glutamine synthetase activity by tabtoxin was observed only when the protease inhibitor phenylmethylsulfonyl fluoride was omitted from the extraction buffer. With this compound in the buffer, only about half as much inhibition of glutamine synthetase was obtained (data not shown). These data indicate that yeast, like plants (35), must activate tabtoxin by proteolytic cleavage and that the peptidase(s) necessary for this activation is present in yeast. This result, combined with the preceding results, indicates that glutamine synthetase is the primary target of MSO and tabtoxin in yeast.

Multiple copies of a glutamine synthetase gene protect *S. cerevisiae* from killing by MSO and tabtoxin. The preceding results suggested that cells overproducing glutamine synthetase will show increased resistance to MSO and tabtoxin. To create such an overproducing strain, we transformed *S. cerevisiae* SJ21 with the recombinant plasmid pJL106 (A. Mitchell, personal communication). The plasmid has a 3.5-kb fragment of *S. cerevisiae* DNA containing *GLN1*, the glutamine synthetase gene, cloned in the *Bam*HI site of YEp24. As a control we used SJ21 transformed with YEp24.

Transformed cells were selected in two ways. First, since pJL106 and YEp24 carry the *URA3* gene that is able to complement the *ura3-52* mutation of strain SJ21, we selected Ura^+ transformants on uracil-deficient medium. Second, we selected transformants directly for resistance to 1 mM MSO (MSO^r) by the plate overlay technique described in Materials and Methods.

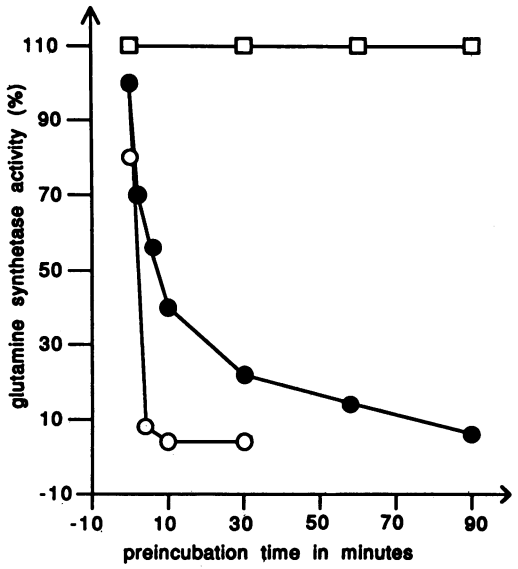

FIG. 2. Inhibition of glutamine synthetase activity by MSO and tabtoxin. *S. cerevisiae* SJ21 was grown in GGlt medium to an A_{600} of 1. Cell extracts were made as described in Materials and Methods, except that phenylmethylsuflonyl fluoride was not included in the extraction buffer. Extracts were preincubated at 30°C with tabtoxin or MSO for the times shown, and then glutamine synthetase transferase activity was measured over a 30-min period. Control extracts were preincubated for various times without MSO or tabtoxin before glutamine synthetase activity was measured. Symbols: □, control; ○, 1 mM MSO; ●, 1.2 mM tabtoxin.

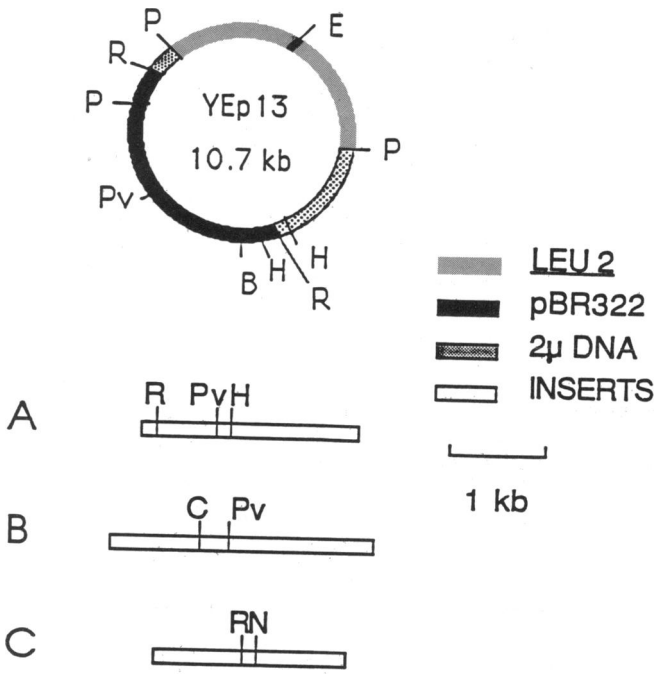

FIG. 3. Restriction map of MSO plasmids. Plasmids were isolated from an *S. cerevisiae* genomic library made by inserting partially *Sau*3A digested DNA from *S. cerevisiae* D13-1A (33) into the *Bam*HI site of the yeast shuttle vector YEp13. A, B, and C show the insert regions of pMSO2, pMSO5, and pMS06, respectively. In all cases the *Bam*HI site was lost because of joining to a *Sau*3A end. The orientation of the yeast DNA insert relative to the vector is as shown. YEp13 carries the pBR322 genes for ampicillin and tetracycline resistance, the *LEU2* gene from *S. cerevisiae*, and the *Saccharomyces* 2μm plasmid region allowing autonomous replication in yeast. Abbreviations: R, *Eco*RI; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; N, *Nru*I.

Plasmid pJL106 gave 4,700 Ura^+ transformants and 3,100 MSO^r transformants per μg of vector DNA. The parent plasmid YEp24 gave 4,000 Ura^+ transformants per μg of DNA and no MSO^r transformants, as expected. Twenty Ura^+ and 20 MSO^r pJL106 transformants were colony purified on selective plates and then spotted on a plate with the opposite selective condition. The 20 MSO^r transformants were all Ura^+, and of 20 Ura^+ transformants, 18 were MSO^r. These data strongly suggest that the two phenotypes are due to pJL106.

To calculate the degree of resistance to MSO and tabtoxin, we determined the concentration of drug that inhibited growth by 50% (ID_{50}). Cells having multiple copies of the glutamine synthetase gene [SJ21(pJL106) transformants] had a 12- to 15-fold increase in resistance to MSO and tabtoxin and overproduced glutamine synthetase activity sixfold (Table 1). This increased level of enzyme activity was directly related to plasmid copy number, which we determined to be 4 to 6 copies per cell (data not shown). These data supported the hypothesis that *S. cerevisiae* with multiple copies of *GLN1* would overproduce glutamine synthetase and become increasingly resistant to MSO and tabtoxin.

Selection of yeast genes that confer MSO resistance. To see whether any yeast genes besides *GLN1* would confer MSO and tabtoxin resistance on their host when carried on a multicopy vector, we transformed strain DC5 with an *S. cerevisiae* clone bank made in YEp13 (Fig. 3). The *leu2* defect in strain DC5 can be complemented by the *LEU2* gene carried on YEp13. Seven thousand Leu^+ transformants were selected and pooled, and then MSO^r cells were selected in two ways. First, 10^4 Leu^+ transformants were grown for 4 days at 30°C in 1 ml of GN medium containing 50 mM MSO.

TABLE 1. Multiple copies of *GLN1* confer MSO and tabtoxin resistance on *S. cerevisiae*[a]

Strain	ID_{50} (mM)		Glutamine synthetase activity[b] (no. of expt)
	MSO	Tabtoxin	
SJ21(YEp24)	0.025	0.08	2.8 ± 0.2 (2)
SJ21(pJL106)	0.38	1.0	17.3 ± 2.14 (3)

[a] Cells were inoculated at a density of 10^3/ml into GNGlt medium containing MSO or tabtoxin at concentrations ranging from 0 to 1 mM. After 19 h of growth, samples were plated on YPD plates to calculate the percentage of colony-forming cells per ml.

[b] Glutamine synthetase transferase activity was measured in cell extracts made from cultures grown to mid-log phase in GGlt medium (derepressed conditions) and is expressed as units (micromoles of γ-glutamylhydroxamate formed per min at 30°C).

TABLE 2. Isolation and classification of plasmids carrying yeast genes that give an MSO-resistant phenotype

Selection conditions[a]	Plasmid(s) isolated[b]
Glutamate (0.1%) + 2.5 mM MSO	pMSO2 (8)
Glutamate (0.1%) + 25 mM MSO	pMSO1 (12), pMSO2 (8), pMSO5 (1)
MSO (2.5 mM)	pMSO2 (4), pMSO5 (1)
MSO (25 mM)	pMSO1 (2), pMSO2 (9), pMSO6 (1)

[a] All selection plates contained 2× yeast nitrogen base, 1% glucose, 0.1% ammonium sulfate, and the nutrients required by strain DC5.

[b] The number of isolates obtained (of 46 analyzed) is shown in parentheses.

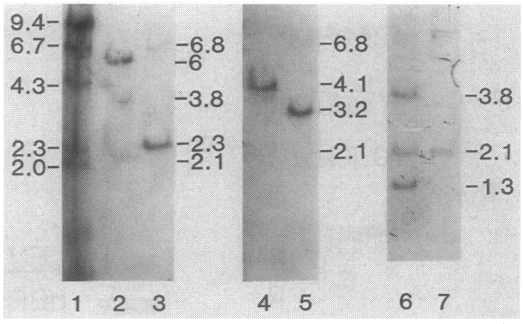

FIG. 4. Southern blot hybridization of MSO genes. DNA from *S. cerevisiae* DC5 was digested with *Eco*RI or *Bam*HI, electrophoresed on a 0.9% agarose gel, and blotted to nitrocellulose. Blots were probed with the ^{32}P-labeled *Eco*RI fragments carrying the pMSO2, pMSO5, or pMSO6 insert-specific probes described in the text. Lanes: 1, *Hin*dIII fragments of lambda end-labeled with ^{32}P; 2, *Eco*RI-cleaved yeast DNA probed with the pMSO2 probe; 3, *Bam*HI-cleaved yeast DNA probed with the pMSO2 probe; 4, *Eco*RI-cleaved yeast DNA probed with the pMSO5 probe; 5, *Bam*HI-cleaved yeast DNA probed with the pMSO5 probe; 6, *Eco*RI-cleaved yeast DNA probed with the pMSO6 probe; 7, *Bam*HI-cleaved yeast DNA probed with the pMSO6 probe. Fragment sizes are indicated (in kilobases).

Samples were plated, and four individual colonies that showed cosegregation of the Leu$^+$ and MSO^r markers were studied. They all contained the plasmid designated pMSO1 (Table 2). In the second procedure, MSO-resistant cells were selected by plating 10^6 Leu$^+$ transformants on plates containing either 2.5 or 25 mM MSO (Table 2). One set of selection plates contained glutamate to facilitate selection of transformants with elevated glutamine synthetase activity. It has been noted that increased glutamine synthetase activity can cause intracellular depletion of glutamate, a substrate for this enzyme, and subsequent growth inhibition. Inhibition can be overcome by adding glutamate to the medium (A. Mitchell, personal communication).

Twenty-five of the largest colonies from each of the four types of plates were colony purified. Total DNA prepared from each isolate was used to transform *E. coli* DG75, with selection for ampicillin resistance. Forty-six samples gave *E. coli* transformants. Plasmid DNA was isolated from each transformant and digested with *Eco*RI. From these data the plasmids could be categorized into four classes (Table 2). Surprisingly, none of the isolates appeared to contain *GLN1*, since none of them contained the 0.8-kb *Eco*RI fragment present in *GLN1* (A. Mitchell, personal communication). Also, we found that while pJL106 complemented the *gln1* defect in strain 745-gln1, as expected for a plasmid carrying the wild-type *GLN1* gene, none of the MSO plasmids complemented *gln1*. These results also ruled out the possibility that one or more of our plasmids coded for a gene that allowed glutamine synthesis by a novel pathway. The *GLN1* gene was probably not selected by these procedures because too few Leu$^+$ cells were examined. Also, only the selection done with 2.5 mM MSO in the presence of 0.1% glutamate (Table 2) would have favored selection of *GLN1*. Since we picked only the largest colonies from these selection plates, we may have overlooked transformants carrying *GLN1*. The other three selection conditions were unfavorable for selecting *GLN1* either because the MSO concentration was too high or because no glutamate was added to the medium and transformants carrying multiple copies of *GLN1* would be starved for glutamate (A. Mitchell, personal communication).

The size of the yeast DNA insert was found from restriction digestions to be 2.3 kb for pMSO2, 2.8 kb for pMSO5, 2.1 kb for pMSO6 (Fig. 3), and 9.3 kb for pMSO1. Because pMSO1 carried a large insert and conferred a low level of drug resistance, we did not characterize it further. Southern blot analysis was used to determine whether the yeast DNA inserts in pMSO2, pMSO5, and pMSO6 were unique. For these experiments, total genomic DNA from *S. cerevisiae* DC5 was cleaved with the restriction endonuclease *Eco*RI or *Bam*HI. When the *Eco*RI-cleaved DNA was hybridized to a ^{32}P-labeled probe specific for the yeast DNA insert in pMSO2 (the fragment extends from the *Eco*RI site in the pMSO2 insert to the *Eco*RI site at the pBR322-2μm junction), a major band of hybridization was observed at about 6 kb and two less intense bands were observed at 3.8 and 2.1 kb (Fig. 4, lane 2). With *Bam*HI-cleaved yeast DNA, this probe detected a major band of 2.3 kb and a minor band of 6.8 kb (Fig. 4, lane 3). With a probe for the pMSO5 yeast insert (an *Eco*RI fragment spanning the pBR322-yeast portion of pMSO5), there was an *Eco*RI band of 4.1 kb (Fig. 4, lane 4) and a *Bam*HI band of about 3.2 kb (Fig. 4, lane 5). A probe representing one-half of the pMSO6 yeast insert (an *Eco*RI fragment going from the *Eco*RI site in the middle of the insert to the *Eco*RI site at the pBR322-2μm junction) hybridized to *Eco*RI bands of 1.3, 3.8, and 2.1 kb (Fig. 4, lane 6), while it hybridized to a *Bam*HI band of 2.1 kb (Fig. 4, lane 7). Since each probe hybridized primarily to a unique *Eco*RI or *Bam*HI chromosomal DNA fragment and because their restriction maps were different (Fig. 3), pMSO2, pMSO5, and pMSO6 probably carry different genes for MSO resistance.

We compared the level of MSO resistance that each MSO plasmid conferred on its host by measuring growth rates in the presence of increasing MSO concentrations (Fig. 5). For these experiments all plasmids were transferred to strain SJ21, which is more sensitive to MSO than strain DC5. Growth of the control strain, SJ21(YEp13), was inhibited 50% by 0.04 mM MSO. Plasmids pMSO1, pMSO2, and

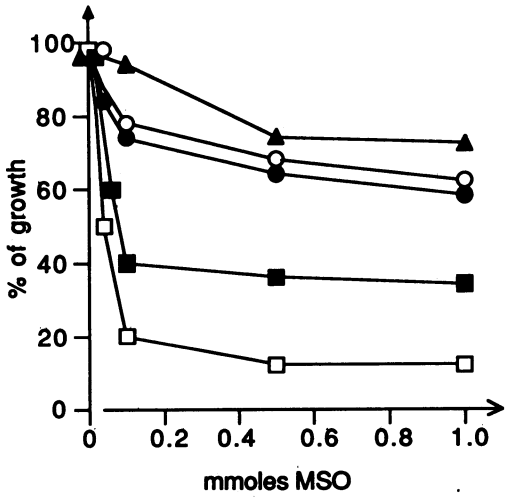

FIG. 5. MSO plasmids confer MSO resistance on yeast. Strain SJ21 was transformed with YEp13 or MSO plasmids. Cultures were started at 10^3 cells per ml in GNGlt medium and grown at 30°C in the presence of increasing concentrations of MSO. After 19 h of growth, samples were plated on YPD plates to calculate the number of colony-forming cells. Growth without inhibitor was considered 100%. Symbols: □, SJ21(YEp13); ●, SJ21(pMSO1); ▲, SJ21(pMSO2); ○, SJ21(pMSO5); ■, SJ21(pMSO6).

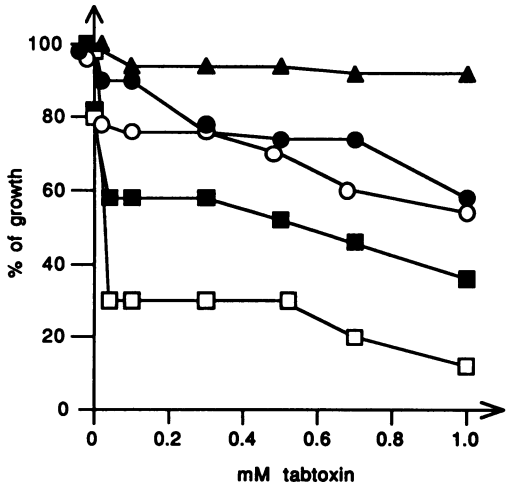

FIG. 6. MSO plasmids confer tabtoxin resistance on yeast. The same procedures were used as described in the legend to Fig. 5 except that a filter-sterilized solution of purified tabtoxin dissolved in water was used in place of MSO. Growth without inhibitor was considered 100%. Symbols: □, SJ21(YEp13); ●, SJ21(pMSO1); ▲, SJ21(pMSO2); ○, SJ21(pMSO5); ■, SJ21(pMSO6).

pMSO5 made their host much more resistant to MSO than the control strain was, but the ID_{50} concentration of drug could not be determined because the growth of these cells was not inhibited as much as the control strain even at the highest drug concentrations used, greater than 5 mM (data not shown). Plasmid pMSO6 made its host at least fivefold more resistant to MSO, the same level of resistance that was conferred by pJL106, which carries *GLN1*. Similar growth experiments were repeated with tabtoxin in place of MSO. pMSO6 made its host at least fivefold more resistant to tabtoxin than the control plasmid YEp13, while pMSO1 and pMSO5 increased the resistance of their hosts even more, and pMSO2 made its host resistant to all concentrations of tabtoxin tested (Fig. 6).

The higher level of host resistance conferred by some MSO plasmids could be due to a larger number of plasmids per cell or to integration of the plasmid into a host chromosome in a manner that would cause increased expression of the plasmid-borne resistance gene(s). Southern blot analysis showed that all strains contained autonomous, not integrated, copies of the plasmid (data not shown) and that there were 3 to 5 plasmid copies per cell, except for YEp13 and pMSO1, which were present at 9 and 11 copies per cell, respectively (data not shown).

Glutamine synthetase activity in MSO-resistant transformants. We measured glutamine synthetase transferase activity in transformants under growth conditions that repressed, derepressed, or gave intermediate levels of enzyme activity (Table 3). This was done to determine whether MSO plasmids conferred MSO resistance by stimulating the level of glutamine synthetase activity, which might occur if an MSO plasmid carried a positive regulatory gene such as *GLN3* (26). Under growth conditions that repressed glutamine synthetase activity (GGln medium), all strains showed low glutamine synthetase activity except SJ21(pJL106) (*GLN1*), SJ21(pMSO2), and SJ21(pMSO5). Under growth conditions that derepressed (GGlt medium) or gave intermediate enzyme levels (GNGlt medium), only strain SJ21(pJL106) showed elevated enzyme activity. These data confirmed that MSO plasmids did not carry *GLN1*, because they did not give elevated glutamine synthetase activity under derepressing conditions, as did pJL106, which carries *GLN1*. Under repressing conditions, strain SJ21(pMSO5) had a slightly elevated level of enzyme activity and strain SJ21(pMSO2) had about a fivefold-higher level of enzyme activity than the control strain. These two plasmids could carry genes that regulate *GLN1* expression, but this seems unlikely since they only elevated enzyme activity under repressing conditions. We also measured the activity of NAD-glutamate dehydrogenase, an enzyme that is coregulated with glutamine synthetase by *GLN3* (26). The glutamate dehydrogenase activity remained normal in all MSO-resistant transformants, indicating that none of the MSO plasmids regulated the level of this enzyme (Table 3).

To explore further the possibility that MSO plasmids carry a gene that regulates *GLN1* in a positive manner, we measured glutamine synthetase activity and mRNA levels in transformed strains after treatment with MSO. We reasoned that if one of the MSO plasmids carried a positive regulatory gene, then after addition of MSO to the culture there should be an increase in glutamine synthetase mRNA because the MSO would inactivate glutamine synthetase, producing a decrease in the intracellular glutamine concentration. The intracellular level of glutamine is sensed in an unknown manner by the positive regulatory gene *GLN3* (26), and the *GLN3* product would stimulate transcription of *GLN1*. The increase in glutamine synthetase mRNA would not be paralleled by an increase in glutamine synthetase activity because of inhibition by MSO.

For this experiment cells were grown in the presence or absence of MSO under conditions that gave intermediate glutamine synthetase levels (GNGlt medium). This growth medium was used because there would be no enzyme inhibition under repressed growth conditions due to the presence of glutamine in the medium, and under derepressed conditions there would be a high basal level of glutamine synthetase mRNA, which might obscure an increased mRNA level. Part of each culture was used for enzyme assays, and part was used for preparation of RNA.

None of the MSO plasmids caused a significant increase in the level of glutamine synthetase mRNA in comparison with

TABLE 3. Glutamine synthetase and NAD-dependent glutamate dehydrogenase activities in yeast transformed with MSO plasmids[a]

Strain	Glutamine synthetase activity[b] (U)			NAD-dependent glutamate dehydrogenase activity[c] (U)		
	Gln	GNGlt	GGlt	GGln	GNGlt	GGlt
SJ21	0.05	0.50	2.28	3	5	38
SJ21(YEp13)	0.04	0.41	2.62	2	4	39
SJ21(pMSO1)	0.07	0.45	2.62	6	2	48
SJ21(pMSO2)	0.28	0.41	2.74	3	1	21
SJ21(pMSO5)	0.12	0.52	3.16	7	4	37
SJ21(pMSO6)	0.07	0.48	3.41	6	6	30
SJ21(YEp24)	0.08	0.51	3.27	9	2	48
SJ21(pJL106)	0.23	1.3	18.1	4	3	47

[a] Saturated cultures were grown in GGln medium with auxotrophic supplements. Cells were then diluted to an A_{600} of 0.02 into GGln (repressed), GNGlt (intermediate), or GGlt (derepressed) medium, grown to an A_{600} of 1.2, and prepared for enzyme assays. The values are the averages from at least three experiments for glutamine synthetase activity and two experiments for NAD-glutamate dehydrogenase activities, except for the cultures grown on GNGlt medium, which represent one experiment.

[b] One unit equals 1 μM of γ-glutamylhydroxamate formed per min at 30°C.

[c] One unit equals 1 nM of NADH oxidized per min per mg of protein at 25°C.

TABLE 4. Glutamine synthetase mRNA in transformants of SJ21[a]

Strain	Relative level of glutamine synthetase mRNA	
	GNGlt	GNGlt + 1 mM MSO
SJ21	1.0	3.0
SJ21(YEp13)	0.6	1.2
SJ21(pMSO1)	0.6	1.1
SJ21(pMSO2)	2.0	2.1
SJ21(pMSO5)	0.9	2.6
SJ21(pMSO6)	1.7	2.8
SJ21(YEp24)	1.7	3.1
SJ21(pJL106)	6.3	17.1

[a] Saturated cultures were grown in GNGlt medium, diluted to an A_{600} of 0.15, and grown in the presence or the absence of 1 mM MSO to an A_{600} of 2. Strains SJ21, SJ21(YEp13), and SJ21(YEp24) are not resistant to MSO and were inoculated at an A_{600} of 0.5 in the medium containing MSO. They were harvested at the same time as the other cultures. The amount of glutamine synthetase mRNA was measured by hybridization to the 0.8-kb internal *Eco*RI fragment of the *GLN1* gene. A fragment of yeast DNA derived from the gene coding for the ribosomal L protein (9) served as an internal control for the amount of mRNA retained on the filter in each sample. All values were normalized to that for strain SJ21 grown in GNGlt medium.

the control strains SJ21, SJ21(YEp24), and SJ21(YEp13) (Table 4). Increased mRNA was only seen in cells transformed with pJL106, which carries *GLN1*. The glutamine synthetase activity values for these strains are presented in Table 5. As expected, all strains had low enzyme activity when MSO was present in the culture medium. However, the MSO-resistant strains all had a slightly higher enzyme level than the nonresistant strains, which must have enabled them to grow in the presence of MSO. We also measured NAD-glutamate dehydrogenase activity and found that it was increased 20- to 80-fold when MSO was present in the culture medium. This result shows that *GLN3* is functioning, since it controls the NAD-glutamate dehydrogenase structural gene, and in the presence of MSO this gene would be turned on due to the decreased intracellular level of glutamine. We conclude from these data that none of the MSO plasmids carries a gene that regulates transcription of *GLN1* in a positive manner, as does *GLN3*.

MSO plasmids block MSO uptake. Cells transformed with MSO plasmids could be resistant to MSO because the drug is prevented from entering the cell. To examine this possibility, we measured the transport of ^{35}S-labeled MSO. Strains were

TABLE 5. Glutamine synthetase and NAD-dependent glutamate dehydrogenase activity in SJ21 transformants grown in the presence or absence of MSO[a]

Strain	Glutamate synthetase activity (U)		NAD-dependent glutamate dehydrogenase activity (U)	
	GNGlt	GNGlt + 1 mM MSO	GNGlt	GNGlt + 1 mM MSO
SJ21	0.49	0.02	5	420
SJ21(YEp13)	0.41	0.07	5	410
SJ21(pMSO1)	0.25	0.18	3	73
SJ21(pMSO2)	0.43	0.15	4	165
SJ21(pMSO5)	0.36	0.19	5	351
SJ21(pMSO6)	0.34	0.10	4	193
SJ21(YEp24)	0.38	0.07	4	390
SJ21(pJL106)	3.3	0.18	5	133

[a] See Table 4, footnote *a*, and Table 3, footnotes *b* and *c*. Strains SJ21, SJ21(YEp13), and SJ21(YEp24) did not go through a division cycle in the presence of MSO.

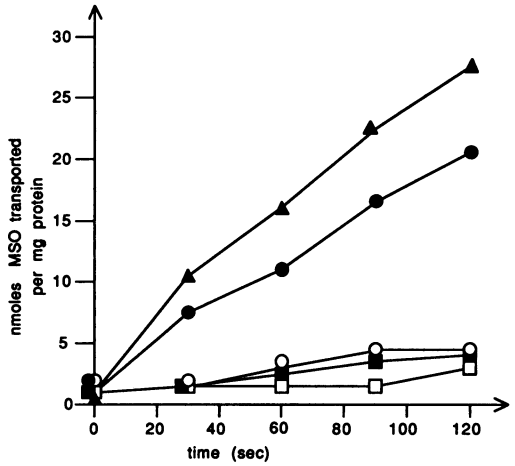

FIG. 7. MSO transport in yeast carrying MSO plasmids. Transport measurements were made as described in Materials and Methods. Symbols: ▲, SJ21(YEp13); ■, SJ21(pMSO1); □, SJ21(pMSO2); ○, SJ21(pMSO5); ●, SJ21(pMSO6).

cultivated in GNGlt medium to match the conditions used for the growth inhibition studies. In strains SJ21(pMSO1), SJ21(pMSO2), and SJ21(pMSO5), the rate of MSO uptake was reduced about 80% compared with that in the control strain, SJ21(YEp13) (Fig. 7). In strain SJ21(pMSO6), the rate of MSO uptake was reduced about 20%. These results indicate that MSO resistance is due to impairment of MSO transport.

The results presented in Fig. 1 suggested that MSO was being transported into the cell by a methionine transporter. Further evidence favoring this interpretation was obtained. First, we found that 0.1 mM methionine inhibited the rate of MSO transport by 50% in strain SJ21, whereas 5 mM glutamine or 5 mM alanine reduced the rate of MSO transport by only 20% (data not shown). Second, we measured the rate of [^{35}S]methionine transport in SJ21 carrying MSO plasmids (Fig. 8) and found that transport was significantly

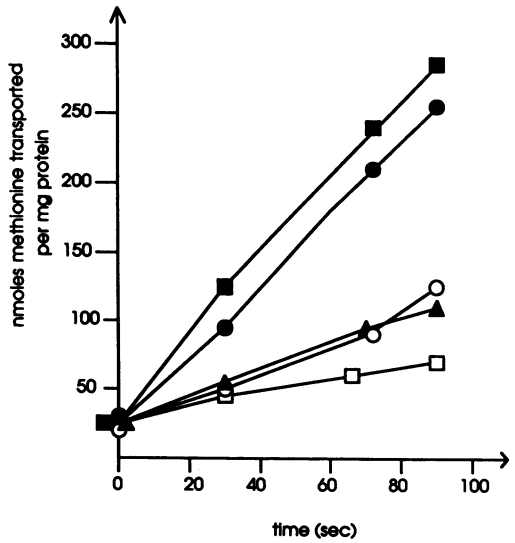

FIG. 8. Methionine transport in yeast carrying MSO plasmids. Transport measurements were made as described in Materials and Methods. Symbols: ■, SJ21(YEp13); ▲, SJ21(pMSO1); □, SJ21(pMSO2); ○, SJ21(pMSO5); ●, SJ21(pMSO6).

reduced by pMSO1, pMSO2, and pMSO5. Only a slight reduction in the rate of methionine transport was produced by pMSO6. These are the same results obtained for MSO transport (Fig. 7), implying that MSO plasmids are reducing the activity of a transporter that transports both methionine and MSO. Third, we measured the K_m for MSO transport and compared it with the K_m for methionine transport. Methionine transport in strain SJ21 occurred by at least two systems, a low-affinity transporter with a K_m of 600 μM and a high-affinity transporter with a K_m of 20 μM (data not shown). These results are similar to published values for methionine transport (11). Similar transport studies with MSO gave a single K_m value of 800 μM so MSO could be using either the high- or the low-affinity methionine transporter or both.

MSO could also be transported by the GAP (12). Transport by GAP can be measured by using citrulline (28). If MSO was transported by GAP, we would expect citrulline transport to be reduced by MSO plasmids, whereas citrulline transport should be normal if MSO is transported by the methionine-specific transporters. We found that the rate of transport of [^{14}C]citrulline was not affected by MSO plasmids (data not shown). We conclude that MSO plasmids are not affecting the activity of GAP. MSO is not transported by this transporter to any large extent under the experimental conditions used here.

DISCUSSION

The data presented here demonstrate that *S. cerevisiae* can be made resistant to MSO and tabtoxin by overproducing glutamine synthetase, the enzyme target of these two drugs. Overproduction of enzyme (Table 1) was achieved by placing the glutamine synthetase gene, *GLN1*, on a yeast shuttle vector, YEp24, that was present at an average of 3 to 5 copies per cell. Other cell types, including mouse 3T3 cells (36) and Chinese hamster ovary cells (29), have been shown to be resistant to MSO because of amplification of their glutamine synthetase gene and consequent overproduction of enzyme activity. In these cases resistant cells were selected after repeated subculturing in the presence of increasing concentrations of MSO. We have not tried to select yeast cells for increased drug resistance, but it should be possible to do so.

By using MSO resistance as a selection procedure, we were able to isolate *S. cerevisiae* genes that make yeast resistant to MSO. The genes, represented by plasmids pMS01, pMS02, pMS05, and pMS06, are not structural genes coding for glutamine synthetase, because they did not complement a *gln1*-defective strain. Plasmids pMS02, pMS05, and pMS06 probably carry unique resistance genes. This conclusion is based on (i) restriction site mapping (Fig. 3), (ii) Southern blotting (Fig. 4), and (iii) the drug resistance level conferred on yeast cells by each plasmid (Fig. 5 and 6). However, our data do not exclude the possibility that these plasmids carry the same resistance gene. Further work, including subcloning, transcription mapping of, and sequencing of the DNA that confers MSO resistance, should resolve the issue of whether the MSO plasmids carry identical or unique resistance genes.

Our results demonstrate that all four MSO plasmids made their host resistant to MSO by reducing the rate of MSO transport across the plasma membrane. To our knowledge this is the first report of MSO resistance resulting from a reduced rate of intracellular drug transport. Several results suggest that MSO is using one or both of the two methionine permeases, rather than GAP, some other amino acid-specific permease, or another type of permease. First, methionine was the only amino acid that reduced the rate of MSO transport. Second, methionine and glutamine were the only amino acids that prevented growth inhibition by MSO. Third, MSO plasmids reduced the rate of transport of both MSO and methionine. Finally, MSO plasmids did not affect the rate of citrulline transport, indicating that they were not reducing GAP activity, which they would be doing if MSO was transported by GAP. It is not clear from our results whether MSO is transported by the low- or the high-affinity methionine transporter (11) or by both. Use of a *METP*-defective strain (11), which lacks the high-affinity transporter, might help to resolve this issue.

There are three obvious ways in which MSO plasmids could be reducing MSO transport. They could be (i) preventing transcription of the permease structural gene, (ii) preventing translation of the permease mRNA, or (iii) inactivating the permease. These three mechanisms are not mutually exclusive, and each MSO plasmid could be using a different one. It is not possible to propose a more detailed model for the function of MSO plasmids because the regulation of methionine permease activity (reviewed in Cooper [4]) and other methionine-related enzymes (reviewed in reference 16) are not understood. Also, one or more MSO plasmids could be influencing methionine permease(s) activity by an indirect affect such as by changing the size of intracellular methionine pools (reviewed in reference 16, p. 249). Inhibition of MSO and tabtoxin transport probably requires multiple copies of the resistance genes, because the parental strain, D13-1A, from which the resistance genes were isolated is very sensitive to each drug and probably has only one copy of each of the genes. Also, YEp13, the vector used in these experiments, has a 2μm origin of replication and is present in multiple copies per cell. Our measurements ranged from 3 to 11 copies per cell. Further experiments with the MSO genes on single-copy vectors will be needed to verify that drug resistance is due to increased gene dosage.

Since methionine is part of the aspartate family of amino acids and since some genes in this family are coregulated (16), it is possible that MSO plasmids regulate other permeases besides the methionine permease. They could regulate nonpermease genes and enzyme activities also. Such coregulation could explain our results for tabtoxin. Since the toxic effects of tabtoxin were not prevented by methionine, it is likely that tabtoxin is not transported by the same methionine permease that transports MSO. If the hypothetical tabtoxin permease was coregulated with the methionine permease by MSO plasmids, then our data would be readily explained. We did not analyze tabtoxin transport directly because of the difficulty and expense of making radioactive tabtoxin. Because of this, we cannot eliminate the possibility that the tabtoxin resistance phenotype conferred by MSO plasmids is due to some mechanism other than exclusion from the cell.

One of our initial reasons for undertaking this research was to gain insight into the mechanism of wildfire disease resistance in *N. longiflora* and to develope procedures for constructing tobacco strains that are resistant to wildfire. Based on our results with *S. cerevisiae*, we would predict that overproduction of glutamine synthetase activity in tobacco would lead to MSO, tabtoxin, and wildfire disease resistance. However, recent studies by Donn et al. (6) suggest that overproduction of glutamine synthetase activity in alfalfa cells does not yield MSO resistance. They selected alfalfa cells in culture that were 20- to 100-fold more resistant

than wild-type cells to the nonselective herbicide L-phosphinothricin, a competitive inhibitor of glutamine synthetase. The 3- to 7-fold overproduction of glutamine synthetase activity in their resistant cell lines was shown to correspond to a 4- to 11-fold amplification of one glutamine synthetase gene. It is not clear why their cell lines failed to show increased resistance to MSO. It may be that MSO has another target in alfalfa besides glutamine synthetase, that higher levels of enzyme are necessary for resistance, or that one of the noncytoplasmic glutamine synthetases in alfalfa is particularly sensitive to MSO and not to phosphinothricin. Without a better understanding of how MSO resistance genes block MSO and tabtoxin transport into yeast, we cannot predict whether they would confer MSO, tabtoxin, or wildfire resistance on tobacco; in some cases they might.

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