JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 157–162 Vol. 179, No. 1
0021-9193/97/$04.00+0

Expression of Bacterial *mtlD* in *Saccharomyces cerevisiae* Results in Mannitol Synthesis and Protects a Glycerol-Defective Mutant from High-Salt and Oxidative Stress

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Received 16 August 1996/Accepted 16 October 1996

Polyols, or polyhydroxy alcohols, are produced by many fungi. *Saccharomyces cerevisiae* produces large amounts of glycerol, and several fungi that cause serious human infections produce D-arabinitol and mannitol. Glycerol functions as an intracellular osmolyte in *S. cerevisiae*, but the functions of D-arabinitol and mannitol in pathogenic fungi are not yet known. To investigate the functions of mannitol, we constructed a new mannitol biosynthetic pathway in *S. cerevisiae*. *S. cerevisiae* transformed with multicopy plasmids encoding the mannitol-1-phosphate dehydrogenase of *Escherichia coli* produced mannitol, whereas *S. cerevisiae* transformed with control plasmids did not. Although mannitol production had no obvious phenotypic effects in wild-type *S. cerevisiae*, it restored the ability of a glycerol-defective, osmosensitive *osg1-1* mutant to grow in the presence of high NaCl concentrations. Moreover, *osg1-1* mutants producing mannitol were more resistant to killing by oxidants produced by a cell-free H_2O_2-$FeSO_4$-NaI system than were controls. These results indicate that mannitol can (i) function as an intracellular osmolyte in *S. cerevisiae*, (ii) substitute for glycerol as the principal intracellular osmolyte in *S. cerevisiae*, and (iii) protect *S. cerevisiae* from oxidative damage by scavenging toxic oxygen intermediates.

Polyols, or polyhydroxy alcohols, are produced by fungi and a wide range of other organisms. One important physiologic function ascribed to these compounds is that they serve as intracellular osmolytes or compatible solutes that protect against osmotic shock (11, 29). *Saccharomyces cerevisiae* responds to osmotic stress by increasing the synthesis and accumulation of glycerol (2, 19). A number of recent reports describe components of a mitogen-activated protein kinase cascade that is involved in the high-osmolarity glycerol signal transduction pathway (3, 18). Also, an *S. cerevisiae* mutant that could not grow at high osmolarity was deficient in *sn*-glycerol-3-phosphate dehydrogenase (NAD^+) (GPD) and in glycerol production, and a single copy of the gene encoding GPD (*GPD1*) restored GPD activity, glycerol production, and osmotolerance to wild-type levels (16). Finally, disruption of *GPD1* in *S. cerevisiae* and of a similar gene (*DAR1*) in *Saccharomyces diastaticus* resulted in decreased glycerol production and increased sensitivity to osmotic stress (1, 6, 25). Thus, glycerol functions as an intracellular osmolyte in *Saccharomyces* species.

Several fungi that cause serious human infections also produce large amounts of acyclic polyols. For example, *Candida albicans* produces the five-carbon polyol D-arabinitol in culture and in infected animals and humans (13, 27), and *Cryptococcus neoformans* and *Aspergillus fumigatus* produce the six-carbon polyol mannitol in culture and in infected animals (26, 28). Little is known about the functions of polyols other than glycerol in fungi. Specifically, it is not known if polyols other than glycerol function as intracellular osmolytes or if they contribute to virulence. To address these issues, we isolated and analyzed a *C. neoformans* mutant that underproduces and underaccumulates mannitol. This mutant was more susceptible to osmotic and heat stress than was its wild-type parent, was hypersusceptible to oxidative killing by normal human neutrophils and by cell-free oxidants, and was hypovirulent in mice (4, 5). These results suggested that mannitol functions in *C. neoformans* as an intracellular osmolyte and antioxidant and that its production is required for wild-type virulence. These hypotheses have not yet been examined directly because the biochemistry and genetics of polyol production by *C. neoformans* and other pathogenic fungi have not yet been elucidated.

The *mtlD* gene of *Escherichia coli* encodes mannitol-1-phosphate dehydrogenase, which interconverts fructose-6-phosphate (and NADH) with mannitol-1-phosphate (and NAD^+). When *mtlD* was expressed in transgenic tobacco, it reduced fructose-6-phosphate to mannitol-1-phosphate, after which mannitol-1-phosphate was dephosphorylated to form mannitol (22). The resulting mannitol-producing plants grew well in high-salt media, whereas mannitol-nonproducing control plants did not (23). Thus, the ability to synthesize mannitol was directly responsible for osmotolerance in plants. With this background, we postulated that a similar approach could be used to assess the functions of polyols in fungi. To test this hypothesis, we expressed *E. coli mtlD* in the model organism *S. cerevisiae* and then tested the transformants for their abilities to synthesize and accumulate mannitol. We also examined the transformants for their abilities to synthesize and accumulate mannitol. We also examined the transformants for their abilities to grow in hyperosmolar media and to survive exposure to toxic oxygen intermediates.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* BWG1-7A (*MAT***a** *his4-119 leu2,112 ura3-52*) was obtained from V. F. Kalb (University of Cincinnati). *S. cerevisiae* UTL-7A (*MAT***a** *ura3-52 trp1 leu2-3,112*) and its *osg1-1* derivative, UTL-7AG3, were provided by K. Larsson (University of Göteborg, Göteborg, Sweden [16]). *S. cerevisiae* YPH 252 (*MAT*α *ura3-52 lys2-801 ade2-101 ade2-101 trp1-1* Δ *leu2-1*Δ*his3-*Δ*200*) and YPH 252 *gpd1*Δ*::leu2* were provided by P. Eriksson (University

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of Göteborg [6]). Yeast isolates were maintained on YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar) or in 0.67% yeast nitrogen base without amino acids (Difco, Detroit, Mich.) supplemented with appropriate nutrients and with 2% glucose (minimal glucose medium) or with 2% galactose and 4% raffinose (minimal galactose-raffinose medium). Agar (2%) was added as needed. Liquid cultures were grown in an incubator shaker (30°C, 180 rpm), and optical densities at 600 nm (OD_{600}) were monitored.

E. coli DH5α (10) was used as the plasmid host unless otherwise indicated. *E. coli* MC11 (F′ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* Nal^r) was obtained from M. Carsiotis (University of Cincinnati). Bacterial cultures were grown in Luria-Bertani medium (21). Mannitol-utilizing *E. coli* strains were identified by their ability to produce red or pink colonies on MacConkey agar base (Difco) plus 1% mannitol.

Plasmids. pBluescript II SK (+) was from Stratagene (LaJolla, Calif.), and pYES2 and pCRII were from Invitrogen (San Diego, Calif.). pGEMD3.6 (which consists of *E. coli mtlA* and *mtlD* on a 3.6-kb *Hin*dIII fragment in pGEM3Z [Promega, Madison, Wis.]) and pGEMD (which consists of *E. coli mtlD* on a 1.5-kb *Nsi*I-*Pst*I fragment in pGEM3Z) were provided by W. G. Niehaus (Virginia Polytechnic Institute, Blacksburg [12]). pMAK705 was provided by S. R. Kushner (University of Georgia, Athens [9]). *E. coli* was transformed according to a modified calcium chloride method (21), and *S. cerevisiae* was transformed by a modified lithium acetate method (7).

Construction of a stable *mtlD* mutant of *E. coli*. A stable *mtlD* mutant of *E. coli* MC11 was constructed as follows. First, a frameshift mutation was introduced at nucleotide 375 of the *mtlD* coding sequence by digesting pGEMD with *Kpn*I, filling in the 4-bp 3′ overhangs with Klenow fragment of DNA polymerase, and rejoining the resulting blunt ends with T4 DNA ligase. The resulting mutant version of *mtlD* was digested with *Hin*dIII and *Pst*I, and the gel-purified fragment was subcloned into *Hin*dIII- and *Pst*I-digested pMAK705 (which contains a heat-sensitive replicon). The resulting plasmid was used to replace the wild-type *mtlD* of *E. coli* MC11 by homologous recombination, as described by Hamilton et al. (9). The resulting mutant strain (*E. coli* VC-1) showed a rate of reversion to mannitol prototrophy of less than 1 in 10^7.

Construction of expression plasmid pY*mtlD*. PCR was used to amplify the *E. coli mtlD* coding sequence from pGEMD. The 5′ oligonucleotide primer was 5′-AAAGCCTCACCCCAGCCTCTC-3′, and the 3′ oligonucleotide primer was 5′-TGTTATTCGGGCGCAGGGTGTC-3′. The 1,215-bp PCR product was ligated into TA cloning vector pCRII (Invitrogen), yielding plasmid pCR-*mtlD*. pY*mtlD* was constructed by subcloning the *Not*I-*Hin*dIII fragment of pCR-*mtlD* into yeast expression plasmid pYES2 (Invitrogen) that had been digested with the same enzymes.

Enzyme assay. Mannitol-1-phosphate dehydrogenase activity was measured according to the method of Novotny et al. (20). *E. coli* VC-1 with or without plasmid pY*mtlD* was grown to early stationary phase in Luria-Bertani medium plus 0.5% mannitol with or without ampicillin, and the cells were collected by centrifugation. The cells were washed three times and resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride; then the cells were passed twice through a French pressure cell (10,000 lb/in^2), and the supernatant obtained by centrifugation at 16,000 × *g* for 45 min and then at 100,000 × *g* for 180 min was used for enzyme assay. The reaction mixture contained 0.1 M Tris hydrochloride (pH 9), 1 mM NAD^+, and supernatant in a total volume of 1 ml. The A_{340} was monitored until no further increase occurred; then 10 mM mannitol-1-phosphate was added, and the change in A_{340} over time was measured.

S. cerevisiae UTL-7AG3 with or without pY*mtlD* was grown to stationary phase in minimal galactose-raffinose medium. The cells were harvested by centrifugation, washed in 50 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin (Boehringer Mannheim) per ml, and 1 μg of pepstatin A (Sigma) per ml, and disrupted by passage through a French pressure cell at 14,000 lb/in^2. The enzyme activity was measured as described above for *E. coli*.

Northern blotting. Total RNA was isolated from cells of *S. cerevisiae* UTL-7AG3, pYES2-transformed UTL-7AG3, and pY*mtlD*-transformed UTL-7AG3 by hot-phenol extraction as described by Köhrer and Domdey (15). Equal amounts of total RNA (1 μg) were separated by agarose gel electrophoresis and transferred to nylon, and mannitol-1-phosphate dehydrogenase mRNA was detected with a 1,215-bp *mtlD* probe that had been labeled with digoxigenin (Genius System; Boehringer Mannheim) according to the manufacturer's instructions. The electrophoresis of RNA, its transfer to a nylon membrane, its hybridization with labeled probe, and detection of mRNA were performed according to standard protocols (21).

Mannitol and glycerol measurements. Total and intracellular mannitol was measured in log- and stationary-phase *S. cerevisiae* cultures by gas chromatography (GC). Briefly, cell-free filtrates or supernatants of cultures that had been heated to 100°C for 10 min to release intracellular mannitol were deproteinized and dried, the trimethylsilyl ether derivatives were formed, and mannitol was measured by GC as previously described (26). The difference between the mannitol concentrations of the heated and unheated samples was considered the intracellular mannitol concentration. That the peaks of interest indeed represented mannitol was confirmed by GC-mass spectrophotometry (GC-MS) as previously described (26) and by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR). For ^{13}C NMR analyses, heated cell suspensions were concentrated in vacuo and resuspended in deionized water, ^{13}C NMR spectra were obtained with a Bruker AC-250 spectrometer operating at a field frequency of 62.89 MHz, and these spectra were compared to those of authentic mannitol. Dimethyl sulfoxide was added as an internal standard; its chemical shift was set at 39.5 ppm. Total glycerol in cell extracts was determined enzymatically with a commercial kit (Boehringer Mannheim).

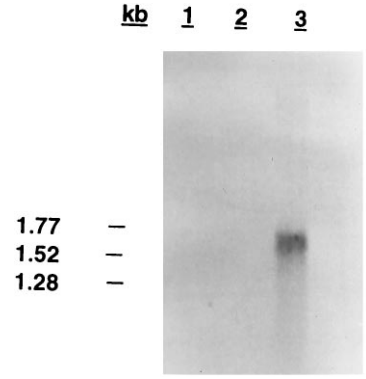

FIG. 1. Northern analyses of *mtlD* expression. Equal amounts of total RNAs (1 μg) from *S. cerevisiae* UTL-7AG3 (lane 1), *S. cerevisiae* UTL-7AG3 transformed with control plasmid pYES2 (lane 2), and *S. cerevisiae* UTL-7AG3 transformed with pY*mtlD* (lane 3) were separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with digoxigenin-labeled *mtlD*. The 1,600-bp product detected in pY*mtlD*-transformed *S. cerevisiae* UTL-7AG3 cells corresponds to the expected *GAL1-mtlD-CYC1* transcription product of pY*mtlD*.

Salt sensitivity. To determine the effects of transformation with pY*mtlD* on salt sensitivity, *S. cerevisiae* UTL-7A, UTL-7AG3, and pYES2- or pY*mtlD*-transformed UTL-7AG3 cells were grown for 24 to 72 h (30°C, 180 rpm) in minimal galactose-raffinose medium (supplemented with uracil when appropriate). These cultures were used to inoculate YPD agar supplemented with 0 to 2.5 M NaCl. Salt sensitivity or resistance was monitored by appearance of growth on YPD agar. Subsequently, *S. cerevisiae* YPH 252 *gpd1*Δ::*leu2* was also tested for salt sensitivity in the presence or absence of pY*mtlD*.

Susceptibility to oxidants. The cell-free microbicidal system of Klebanoff (14) was used to test the susceptibility of mannitol-synthesizing *S. cerevisiae* cells to cell-free oxidants. *S. cerevisiae* UTL-7A, UTL-7AG3, and pY*mtlD*-transformed UTL-7AG3 were grown in minimal galactose-raffinose medium with or without uracil for 48 h at 30°C. In polystyrene tubes (12 by 75 mm), 10^5 yeast cells, 20 mM sodium acetate buffer (pH 5.5), 10 μM H_2O_2, 10 μM $FeSO_4$ and 2 to 10 μM NaI were mixed in a total volume of 500 μl. The tubes were incubated at 30°C for 60 min on a rotary shaker (60 rpm). The cell suspensions were then serially diluted and plated on YPD agar plates to determine CFU.

RESULTS

Properties of plasmid pY*mtlD*. The ability of pY*mtlD* to express catalytically active mannitol-1-phosphate dehydrogenase was first tested in the stable mannitol auxotroph *E. coli* VC-1. *E. coli* VC-1 cells transformed with pY*mtlD* utilized mannitol (i.e., they produced pink colonies on MacConkey-mannitol agar), and lysates prepared from these cells contained abundant mannitol-1-phosphate dehydrogenase catalytic activity (56 U/mg of soluble protein). In contrast, *E. coli* VC-1 cells transformed with the control plasmid pYES2 did not utilize mannitol, and they contained no measurable enzyme activity. Thus, the recombinant protein encoded by pY*mtlD* was a functional mannitol-1-phosphate dehydrogenase.

Expression of *mtlD* in *S. cerevisiae*. Lysates of *S. cerevisiae* BWG1-7A and UTL-7AG3 cells contained high levels of NAD reductase activity in the presence or absence of the mannitol-1-phosphate. Nevertheless, we analyzed lysates of pY*mtlD*- and pYES2-transformed *S. cerevisiae* UTL-7AG3 cells that had been grown on galactose-raffinose medium, and we found no consistent evidence of mannitol-1-phosphate dehydrogenase catalytic activity. However, pY*mtlD*-transformed *S. cerevisiae* UTL-7AG3 cells that had been grown on galactose-raffinose

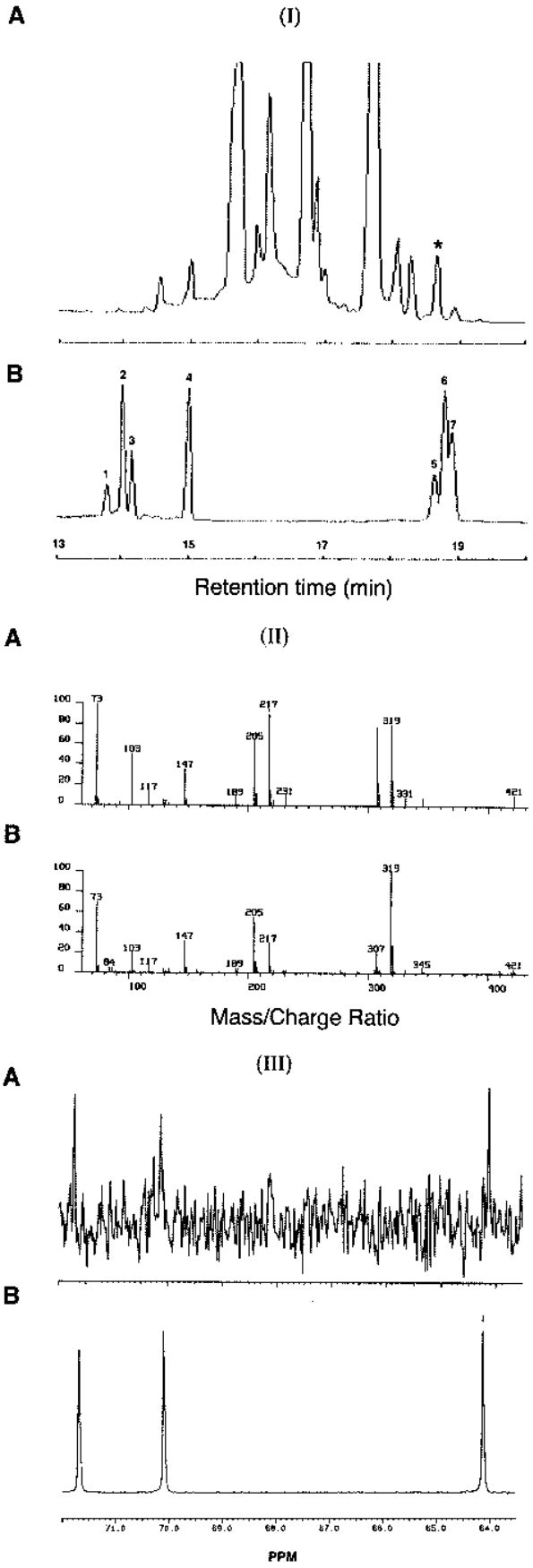

FIG. 2. Mannitol production by pY*mtlD*-transformed *S. cerevisiae*. The trimethylsilyl ether derivatives of an extract of pY*mtlD*-transformed *S. cerevisiae* BWG1-7A cells (IA) and a standard polyol mixture (IB) were analyzed by capillary GC. The retention time of the peak labeled with an asterisk was identical to that of mannitol (peaks in panel IB were as follows: 1, xylitol; 2, D-arabitol; 3, ribitol; 4, internal standard α-methyl D-mannoside; 5, mannitol; 6, sorbitol; and 7, galactitol). When an extract of pY*mtlD*-transformed *S. cerevisiae* cells was analyzed by GC-MS, the electron impact mass spectrum of the peak coeluting with mannitol (IIA) was essentially identical to that of the trimethylsilyl derivative of mannitol (IIB). Finally, ^{13}C NMR spectra of an extract of pY*mtlD*-transformed *S. cerevisiae* BWG1-7A cells (IIIA) and of authentic mannitol (IIIB) showed the expected shifts of 64.1, 70.1, and 71.7 ppm.

medium contained abundant *mtlD* mRNA, whereas yeast cells without plasmid and yeast cells transformed with pYES2 did not (Fig. 1). This indicated that bacterial *mtlD* can be overexpressed in *S. cerevisiae* under *GAL1* regulation.

Mannitol and glycerol production by *S. cerevisiae*. We next examined if the presence of abundant *mtlD* mRNA, and presumably also functional mannitol-1-phosphate dehydrogenase, would result in mannitol biosynthesis, as has been described for transgenic tobacco plants. Five randomly selected independent clones of pY*mtlD*-transformed *S. cerevisiae* BWG1-7A cells were grown to log phase in minimal raffinose-galactose medium; the cell suspensions were then heated to release intracellular polyols, and the cell-free supernatants were analyzed by GC. A peak with the same retention time as mannitol was observed in all five extracts of the pY*mtlD*-transformed cells but not in extracts of pYES2-transformed controls. That the peak of interest represented mannitol was confirmed by GC-MS and by ^{13}C NMR (Fig. 2).

Since glycerol is the principal intracellular osmolyte in *S. cerevisiae*, we also expressed *mtlD* in *S. cerevisiae* UTL-7AG3 (an osmosensitive, glycerol-defective *osg1-1* mutant). Mannitol production by pY*mtlD*-transformed *S. cerevisiae* UTL-7AG3 cells grown in galactose-raffinose medium was also demonstrated by GC, by GC-MS, and by ^{13}C NMR (data not shown). Mannitol was produced during the logarithmic growth and after the culture reached stationary phase (Table 1). Cultures grown in glucose medium contained less mannitol (5.1 μg/ml at 48 h) than those grown on galactose-raffinose, which indicated that heterologous *mtlD* expression was under *GAL1* regulation. Also, mannitol was not detected extracellularly in cell-free culture supernatants until after 48 h, which indicated that actively growing *S. cerevisiae* transformants retained mannitol intracellularly.

Total glycerol concentrations in *S. cerevisiae* UTL-7A and UTL-7AG3 cells were 4 and 2.6 μg/ml of culture volume, respectively. No change in glycerol content was detected when *S. cerevisiae* was transformed with pY*mtlD*.

TABLE 1. Mannitol biosynthesis in *S. cerevisiae* UTL-7AG3 transformed with pY*mtlD*[a]

Time (h)	OD_{600}	Total mannitol (μg/ml)[b]
24	5.0	8
48	6.83	20
96	6.85	37
144	7.0	44

[a] Cultures were grown in YNB-raffinose-galactose medium without uracil in an incubator at 30°C and 180 rpm using a starter inoculum with an OD_{600} of 0.1. The values are the averages of the data from two experiments.

[b] Total mannitol represents mannitol per milliliter of heated culture.

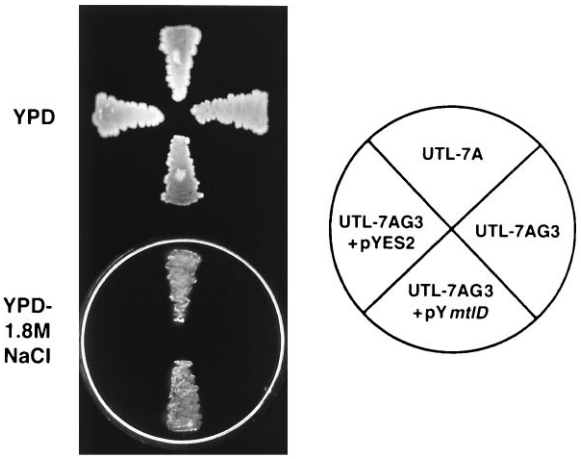

FIG. 3. Growth of *S. cerevisiae* strains on YPD agar with 1.8 M NaCl. The *S. cerevisiae* strains were cultured to log phase in liquid minimal galactose-raffinose medium; aliquots were then streaked on YPD agar or YPD agar supplemented with 1.8 M NaCl, and the plates were incubated at 30°C for 7 days. All strains tested grew well on YPD agar, but only wild-type *S. cerevisiae* UTL-7A and pY*mtlD*-transformed *S. cerevisiae* UTL-7AG3 were able to grow in the presence of 1.8 M NaCl.

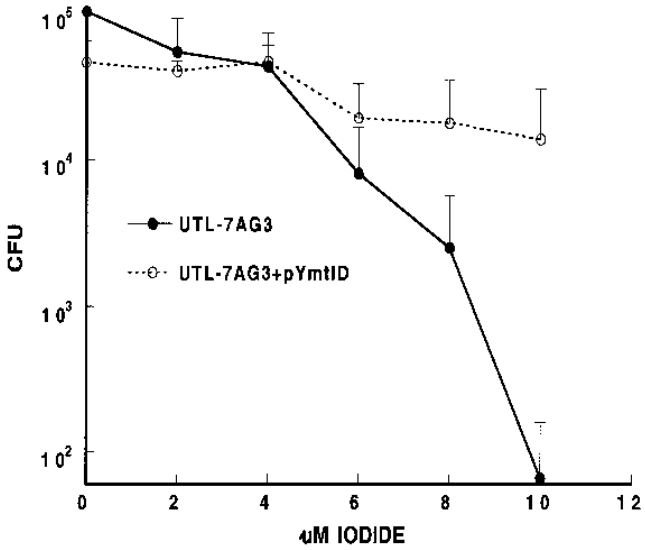

FIG. 4. Susceptibility of *S. cerevisiae* to oxidative killing. Viable cells (10^5) of *S. cerevisiae* UTL-7AG3 or pY*mtlD*-transformed UTL-7AG3 were exposed for 60 min to 10 μM H_2O_2, 10 μM $FeSO_4$, and 0 to 10 μM iodide, and CFU were determined. pY*mtlD* protected *S. cerevisiae* UTL-7AG3 cells from being killed by the oxidants generated in the presence of 6 to 10 μM iodide ($P < 0.05$). The data shown are the means ± the standard deviations (error bars) of values obtained from three experiments.

Phenotypic consequences of *mtlD* expression. Transformation of *S. cerevisiae* BWG1-7A with pY*mtlD* had no obvious phenotypic consequences. Also, *S. cerevisiae* UTL-7A, UTL-7AG3, and pY*mtlD*- or pYES2-transformed UTL-7AG3 did not differ in morphology, growth characteristics, or glycerol production when cultured on conventional YPD agar or in liquid YPD.

Suspensions of *S. cerevisiae* UTL-7A, UTL-7AG3, pYES2-transformed UTL-7AG3, and pY*mtlD*-transformed UTL-7AG3 cells were spotted on YPD agar supplemented with increasing amounts of NaCl to determine salt sensitivity. *S. cerevisiae* UTL-7A grew on media containing as much as 2.25 M NaCl. *S. cerevisiae* UTL-7AG3 and pYES-transformed UTL-7AG3 showed a few tiny revertant colonies on YPD plus 1.8 M NaCl but no growth on media containing 2.0 M or higher NaCl concentrations. In contrast, pY*mtlD*-transformed *S. cerevisiae* UTL-7AG3 grew as well as UTL-7A on media containing up to 2.25 M NaCl (data not shown). When *S. cerevisiae* UTL-7AG3 and pYES-transformed UTL-7AG3 were streaked on YPD plus 1.8 M NaCl, growth was completely inhibited. In contrast, both UTL-7A and pY*mtlD*-transformed UTL-7AG3 grew well on YPD plus 1.8 M NaCl (Fig. 3).

Finally, we verified the ability of pY*mtlD* to confer salt tolerance on a *GPD1*-disrupted yeast mutant (*S. cerevisiae* YPH 252 *gpd1*Δ::*leu2*). The parent strain, *S. cerevisiae* YPH 252, grew on media containing as much as 1.6 M NaCl, while the mutant *S. cerevisiae* YPH 252 *gpd1*Δ::*leu2* failed to grow on media containing 1.0 M or higher NaCl concentrations. However, pY*mtlD*-transformed *S. cerevisiae* YPH 252 *gpd1*Δ::*leu2* grew well on YPD plus 1.6 M NaCl (data not shown).

Susceptibility to oxidative killing. Oxidants generated with 10 μM H_2O_2, 10 μM $FeSO_4$, and 2 to 10 μM NaI killed nontransformed *S. cerevisiae* UTL-7AG3 cells as well as UTL-7AG3 cells containing pY*mtlD*. However, significantly fewer *S. cerevisiae* UTL-7AG3 cells were killed in the presence of pY*mtlD* than in its absence when the NaI concentrations were 6 to 10 μM (Fig. 4). Indeed, the *S. cerevisiae* UTL-7AG3 cells transformed with pY*mtlD* were as resistant to oxidative killing as were wild-type *S. cerevisiae* UTL-7A cells (data not shown), which indicated that the presence of intracellular mannitol provided protection against killing by HO · and other reactive oxygen intermediates equivalent to the protection afforded by the endogenous polyol glycerol.

DISCUSSION

The major goals of this study were to construct a new mannitol biosynthetic pathway in *S. cerevisiae* and then to use *S. cerevisiae* cells expressing this pathway to study the functions of polyols in fungi. We used the inducible *GAL1* promoter on the multicopy yeast expression plasmid pYES2 to overexpress *E. coli mtlD* in *S. cerevisiae*. pY*mtlD*-transformed *S. cerevisiae* cells contained abundant mannitol-1-phosphate dehydrogenase mRNA, and they synthesized mannitol. Thus, we have extended to fungi the strategy by which Tarczynski et al. (22) redeployed the *E. coli* mannitol-catabolizing enzyme to catalyze the first step in a new mannitol biosynthetic pathway (Fig. 5). That mannitol was generated clearly implies that pY*mtlD*-transformed *S. cerevisiae* cells contained catalytically active recombinant mannitol-1-phosphate dehydrogenase. Our inability to demonstrate this enzyme directly in lysates of pY*mtlD*-transformed *S. cerevisiae* cells was probably due to high background levels of NAD reductase activity and/or to rapid degradation of mannitol-1-phosphate dehydrogenase by intracellular proteases or other mechanisms.

Fungi exposed to increasing external osmolarity generate an osmotic gradient across the cell membrane to readjust cellular volume and turgor pressure. Glycerol accomplishes this task with minimal disturbance of metabolic functions because it is compatible with multiple enzymes, even at very high concentrations (2, 19). The ability to produce mannitol had no obvious phenotypic consequences in a wild-type *S. cerevisiae* strain capable of responding to osmotic stress by synthesizing glycerol. In contrast, the abilities to produce and to accumulate mannitol protected the glycerol-defective and osmosensitive mutant *S. cerevisiae* UTL7-AG3 from osmotic stress. This observation establishes a clear cause-and-effect relationship be-

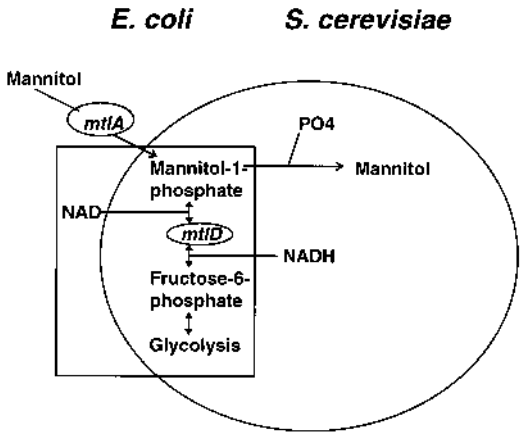

FIG. 5. Mannitol metabolism in *E. coli* and in pY*mtlD*-transformed *S. cerevisiae*. In *E. coli* (rectangular cell), the *mtlA* gene product converts extracellular mannitol to intracellular mannitol-1-phosphate, which is oxidized by the *mtlD* gene product (mannitol-1-phosphate dehydrogenase) to fructose-6-phosphate. In pY*mtlD*-transformed *S. cerevisiae* cells (oval cell), the *mtlD* gene product reduces intracellular fructose-6-phosphate to mannitol-1-phosphate, which is dephosphorylated to yield intracellular mannitol.

tween mannitol biosynthesis and osmotolerance. In addition, it shows that mannitol can substitute for glycerol as the principal osmolyte in *S. cerevisiae*. The amounts of mannitol (20 to 44 μg/ml of culture) synthesized by pY*mltD*-transformed *S. cerevisiae* UTL-7AG3 cells were much less than the amounts of glycerol (0.53 to 2.4 mg/ml of culture) reported in *Saccharomyces* species (1, 25). However, these values were similar to the previously reported wide range of 25 to 500 μg of mannitol/ml of culture in *C. neoformans* (28).

The various acyclic polyols produced by fungi differ only in the length of the carbon chain and in stereochemistry. Our results suggest that different fungal polyols also may be interchangeable in fungi, at least with regard to their osmolyte functions. Therefore, it may be possible to exploit the phenotypic effects in *S. cerevisiae* of new polyol biosynthetic pathways to clone the genes or cDNAs encoding key polyol biosynthetic enzymes in other fungi. For example, it may be possible to isolate the genes or cDNAs encoding mannitol-1-phosphate dehydrogenase in mannitol-producing fungi such as *C. neoformans* or *Aspergillus* species and the corresponding enzyme (D-arabinitol-5-phosphate dehydrogenase) in D-arabinitol-producing fungi such as *C. albicans* (27) by their abilities to confer osmotolerance on *gpd1* mutants such as *S. cerevisiae* UTL-7AG3 or *S. cerevisiae* YPH 252 *gpd1*Δ::*leu2.*

The results of the present study may also have implications relating to pathogenesis. Hydroxyl radicals (HO ·) are potent microbicidal species that attack biomolecules with rate constants of 10^9 M^{-1} s^{-1} or higher. To be effective, HO · scavengers must have high rate constants ($>10^{10}$ M^{-1} s^{-1}) and molar concentrations in vivo (8). Mannitol and ethanol have been widely used as laboratory reagents to scavenge HO · generated by the phagocyte respiratory burst or by cell-free oxidant systems (24). The microbicidal species generated by the H_2O_2-$FeSO_4$-NaI system is believed to be HO · , which then interacts with iodide to form additional toxic products. This interpretation is consistent with the abilities of the HO · scavengers mannitol and ethanol to inhibit killing of bacteria or fungi by the H_2O_2-$FeSO_4$-NaI system (14, 17).

With this background, we reasoned that polyols produced endogenously by fungi should scavenge oxidants generated by the phagocyte oxidative burst and that the ability of a fungus to synthesize and accumulate these polyols should allow the fungi to resist mammalian host defenses. This concept is supported by our recent observation that a mannitol-underproducing *C. neoformans* mutant was less virulent in mice and more susceptible to oxidative killing than was its wild-type parent (4, 5). The present study extends these earlier results in two ways. First, we have shown that the glycerol-defective *S. cerevisiae* mutant UTL-7AG3 is more susceptible than its wild-type parent to HO · and other oxidants generated by the H_2O_2-$FeSO_4$-NaI system. Second, we have shown that the abilities to produce and to accumulate mannitol can protect glycerol-defective *S. cerevisiae* from the deleterious effects of cell-free oxidants. To our knowledge, it has not previously been shown that any endogenously produced polyol functions as a scavenger of HO · or other reactive oxygen intermediates. Finally, the availability of a model organism expressing a functional mannitol biosynthetic pathway should prove useful for defining the mechanisms by which mannitol or other polyols produced by pathogenic fungi scavenge oxidants produced by mammalian phagocytes.

ACKNOWLEDGMENTS

We thank K. Jayasimhulu (Department of Environmental Health, University of Cincinnati) for performing the gas chromatography-mass spectrometry and E. Brooks (Department of Chemistry, University of Cincinnati) for performing the ^{13}C NMR analyses. We also thank K. Larsson and P. Eriksson for providing *S. cerevisiae* strains.

This study was supported by U.S. Public Health Service grant AI-36684 from the National Institute of Allergy and Infectious Diseases and by a grant from the U.S. Department of Veterans Affairs.

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