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Isolation of a *Saccharomyces cerevisiae* Mutant Strain Deficient in Deoxycytidylate Deaminase Activity and Partial Characterization of the Enzyme

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Deoxycytidylate deaminase activity in *Saccharomyces cerevisiae* has been partially characterized. The yeast enzyme was found to exhibit properties similar to those of dCMP deaminases isolated from higher eucaryotes. A mutant strain completely deficient in dCMP deaminase activity was isolated by selection for resistance to 5-fluoro-2′-deoxycytidylate followed by screening for cross sensitivity to 5-fluoro-2′-deoxyuridylate, a potent inhibitor of the yeast thymidylate synthetase. We have designated this new allele *dcd1*. A strain exhibiting an auxotrophic requirement for dUMP was isolated after mutagenesis of a *dcd1 tup7* haploid. Genetic analysis revealed that this auxotrophic phenotype resulted from a combination of the *dcd1* allele and a second, unlinked, nuclear mutation that we designated *dmp1*. This allele, which by itself conveys no readily discernable phenotype, presumably impairs efficient synthesis of dUMP from UDP. The auxotrophic requirement of *dcd1 dmp1 tup7* strains also can be satisfied by exogenous dTMP but not deoxyuridine.

In recent years numerous studies, in both procaryotic and eucaryotic systems, have shown that disturbances in deoxynucleotide biosynthesis are capable of inducing various kinds of genetic change (15). Previous work in our laboratory has shown that inhibition of dTMP synthesis in *Saccharomyces cerevisiae* results in enhanced frequencies of mitotic recombination events, both gene conversion and mitotic crossing over (1, 16). In view of these findings, particularly those involving pyrimidine deoxynucleotides, the need for detailed information on the regulation of deoxynucleotide metabolism has become especially acute. Despite the growing popularity of *S. cerevisiae* as an experimental system, relatively little is known of deoxynucleotide metabolism in this organism. Only two of several enzymes involved in this process, thymidylate synthetase and ribonucleotide reductase, have been characterized previously in *S. cerevisiae* (2, 34).

In most organisms, deoxycytidine nucleotides are shunted into the dTMP biosynthetic pathway via a hydrolytic deamination reaction catalyzed by dCMP deaminase (dCMP aminohydrolase; EC 3.5.4.12). dUMP, the product of this reaction, can be synthesized also from UDP through a series of reactions (reactions 1 to 4) shown in Fig. 1. Thus, dCMP deaminase supplies an alternative source of cellular dUMP. Although this enzyme appears to be common to higher eucaryotes and has been characterized in a variety of bacteria including *Bacillus subtilis* (21), *Lactobacillus acidophilus* (28), and *Staphylococcus aureus* (9), it is not ubiquitous in nature. Both uninfected *Escherichia coli* and *Salmonella typhimurium* lack dCMP deaminase and possess instead distinct dCTP deaminases (23, 24). Since *S. cerevisiae* is similar to both of these bacteria, in the sense that it appears to lack deoxynucleoside kinase activity (4, 25), it remained a distinct possibility that this lower eucaryote also would metabolize deoxycytidylates in a similar manner. To learn more of deoxynucleotide metabolism in *S. cerevisiae*, we sought to determine the means by which deoxycytidylates are deaminated in this organism.

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In this paper, we report (i) preliminary characterization of dCMP deaminase in *S. cerevisiae*, (ii) the isolation of a mutant strain deficient in this activity, and (iii) the characterization of a second mutation which, in combination with the dCMP deaminase deficiency, induces an auxotrophic requirement for dUMP. The results of this study indicate that dCMP deamination alone supplies sufficient dUMP to sustain growth in *S. cerevisiae*. These strains should prove useful in furthering our understanding of the genetic alterations induced by aberrant deoxynucleotide metabolism in this simple eucaryote.

MATERIALS AND METHODS

Strains and media. The haploid strain B500C (*MATα tup7-1*) was provided by L. Bisson, and the properties of the *tup7* allele have been described previously (4). Strain ACM-13, which exhibits a reduced level of nonspecific phosphatase activity, was derived from B500C by selection for resistance to cytosine arabinoside-5′-monophosphate. For tetrad analysis, strain DMPBR2 was constructed by crossing DMPA (*MATα dcd1 dmp1 ilv1-92 tup7-1*) with BR2 (*MATα ade1 leu2-3 his7 tup7-1*, provided by B. A. Kunz). All strains were routinely cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose). When required, nucleotides were added to medium after autoclaving and cooling to 50°C. Sporulation was induced in acetate medium (30).

Chemicals. 5-fluoro-2′-deoxycytidine (FCdR) was purchased from Calbiochem-Behring. The 5′-monophoshate (FdCMP) was prepared from FCdR by the procedure of Tanaka et al. (31) with the following modifications. FdCMP was eluted from a DE52 (Whatman) anion exchange column (2.0 by 21 cm) with a 500-ml linear gradient of NaH_2PO_4 (0 to 0.4 M). Fractions containing FdCMP were pooled and lyophylized, and the nucleotide was extracted from the resulting salt residue by washing several times with 100% methanol and filtering through several layers of Whatman no. 1 filter paper. Methanol was then removed by evaporation, leaving the crystallized nucleotide. The preparation was found to be free of 5-fluoro-2′-deoxyuridylate (FdUMP)

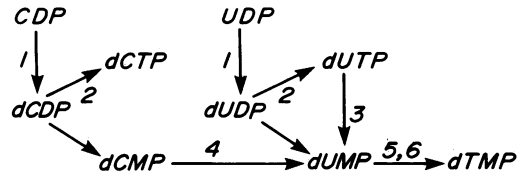

FIG. 1. Enzymes of pyrimidine deoxynucleotide metabolism in *S. cerevisiae*. 1, Ribonucleotide reductase; 2, nucleoside diphosphate kinase; 3, dUTP pyrophosphatase; 4, dCMP deaminase; 5, dihydrofolate reductase; and 6, thymidylate synthetase.

as judged by high-pressure liquid chromatography. All other nucleotides were purchased from Sigma Chemical Co.

Preparation of cell extracts and enzyme assay. For small scale, rapid preparations of dCMP deaminase, single-colony isolates of strains to be tested were grown overnight and used to inoculate medium (600 ml) to a density of about 2×10^6 cells per ml. Cultures were then incubated at 30°C and grown to mid-log phase (1×10^7 to 4×10^7 cells per ml). Cells were harvested by centrifugation at 4°C and washed with ice-cold distilled water. The resulting cell paste was stored at 70°C until use. Upon thawing, 100 μl of water was added, and 1 ml of this dense cell suspension was transferred to a glass test tube. Acid-washed glass beads (type V; Sigma) were then added, and the slurry was vortexed four times, 10 s each time, with cooling on ice during each interval.

The lysate was removed with a Pasteur pipette, transferred to an Eppendorf tube, and centrifuged at 12,900 × *g* for 30 min. The opalescent supernatant was removed and dialyzed for 2 h against two changes of 50 mM Tris-hydrochloride (pH 7.5)–2 mM β-mercaptoethanol–5 mM $MgCl_2$. The resulting crude preparation was then used for assay or stored at −70°C until needed. For large-scale preparation of the enzyme with minimal contaminating phosphatase activity, strain ACM-13 was grown to mid-log phase in 16 liters of YPD medium (containing 1.5 g of KH_2PO_4 per liter) at room temperature with vigorous aeration. Cells were harvested and washed as described above. Ten milliliters of water and 5 g of glass beads were added to ca. 40 g of cell paste. Cells were disrupted by sonication (model W-370, Heat Systems Ultrasonics, Plainview, N.Y.) at 15-s intervals for a total sonication time of 20 min at maximum output. During sonication, the cell suspension was cooled in an ice-ethanol bath. Unbroken cells, mitochondria, and cell debris were removed by centrifugation at 26,500 × *g*. An equal volume of 1.6% streptomycin sulfate (wt/vol) was added to the supernatant with stirring, and precipitated nucleic acids were removed by centrifugation at 12,000 × *g* for 20 min. dCMP deaminase was precipitated by the addition of solid ammonium sulfate (0.3 g/ml) and pelleted by centrifugation at 12,000 × *g* for 20 min. The pellet was suspended in 10 ml of dialysis buffer and desalted on a Sephadex G-100 column (2.0 by 40 cm). Fractions containing dCMP deaminase activity were pooled and stored at −70°C.

dCMP deaminase assays contained, in a total volume of 200 μl, 50 mM Tris-hydrochloride (pH 8.2), 10 mM PP_i, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 40 μM dCTP, and 2 mM dCMP. Enzyme assays were initiated by the addition of protein, incubated for 30 min at 30°C, and terminated by heating to 100°C for 3 min. After cooling on ice, samples were centrifuged at 12,900 × *g*, and 10-μl samples of the supernatant were analyzed by high-pressure liquid chromatography (Beckman model 322). Substrate and product were separated on a Serva Polyol Si100 DEAE anion exchange column, using 0.05 M $NH_4H_2PO_4$ (pH 3.3) as the mobile phase. For assays requiring quantitation of both substrate and product, [^{3}H]dCMP (10 mCi/mmol) was used.

Isolation of a dCMP deaminase mutant. Strain B500C was mutagenized with ethyl methanesulfonate as described by Sherman et al. (30). Mutagenized cells were plated at 10^5 cells per plate on solid YPD medium containing 4 mg of FdCMP per ml and 240 μg of uracil per ml. After 5 days of incubation at 30°C, resistant colonies were picked and streaked onto solid YPD medium containing FdUMP (200 μg/ml) and uracil (240 μg/ml). After 4 days of further incubation at 30°C, isolates exhibiting marked sensitivity to FdUMP were assayed for dCMP deaminase activity.

Isolation of a dUMP auxotroph. Strain EMD1 (*MATα dcd1 tup7-1*) was mutagenized with ethyl methanesulfonate and suspended at 2×10^6 cells per ml in YPD broth containing 200 μg of dUMP per ml. After 12 h of incubation at 30°C, cells were harvested by centrifugation, washed once, and resuspended at a density of 2×10^6 cells per ml in YPD medium containing methotrexate (100 μg/ml) and sulfanilamide (5 mg/ml). After a further 24 h of incubation at 30°C, cells were harvested and washed, and 3×10^3 cells per plate were spread on YPD medium supplemented with 100 μg of dTMP per ml. After 3 days of incubation, colonies were replica plated onto YPD medium, with and without dTMP. Three days later, apparent dTMP auxotrophs were picked and scored for growth on medium containing either folinic acid (100 μg/ml), dUMP (200 μg/ml), or dTMP.

Other methods. Cell counts were determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) with mild sonication before counting. Protein determinations were done by the method of Bradford (6).

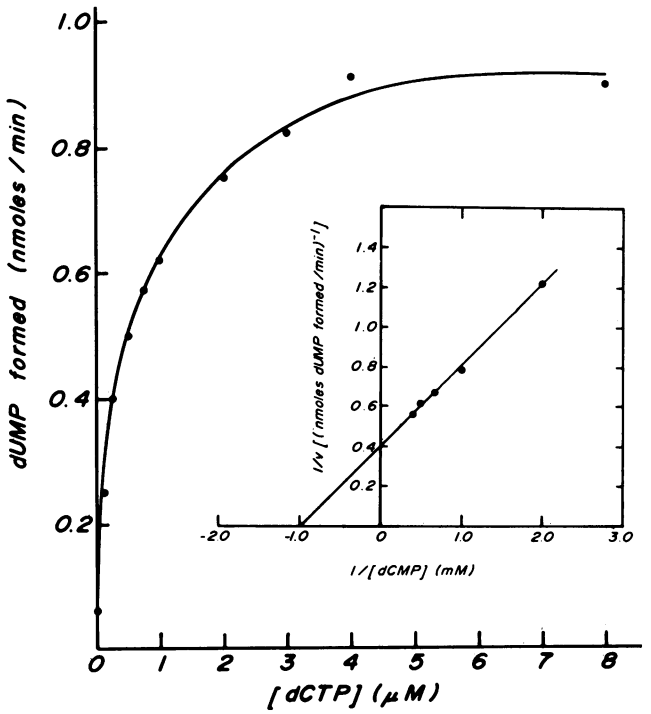

FIG. 2. Activation of dCMP deaminase by dCTP. Inset, Lineweaver-Burk plot.

RESULTS

Characterization of dCMP deaminase activity. Under appropriate conditions, dCMP deaminase activity can be detected in crude lysates of *S. cerevisiae*. Preliminary studies indicated that dialyzed lysates contained sufficient phosphatase activity to interfere seriously with the enzyme assay. This could be overcome, however, by including moderate concentrations of PP_i in the assay buffer. For preliminary kinetic studies, a partially purified preparation of the enzyme was made from a strain that exhibits low levels of intracellular phosphatase activity.

Consistent with the properties of dCMP deaminases isolated from other systems, the yeast dCMP deaminase was activated severalfold by micromolar concentrations of dCTP (Fig. 2). This activation required the presence of Mg^{2+} ions which do not, in the absence of dCTP, greatly affect the activity of the enzyme (data not shown). The apparent K_a for dCTP in the presence of Mg^{2+} was estimated to be ca. 0.8 μM. This value is very similar to apparent K_a values reported for the enzyme isolated from human spleen (10), *B. subtilis* (21), and chicken embryo cells (20). Regression analysis of a Lineweaver-Burk plot revealed an apparent K_m for dCMP of 1.0 mM (Fig. 2), which is also very close to K_m values reported for the human spleen (10), hamster kidney (27), and chicken embryo (20) dCMP deaminases. The yeast enzyme exhibited a broad pH optimum (between pH 7.5 and 9.5) in the presence of saturating amounts of both substrate and effector (data not shown) and in this respect is again similar to both the hamster kidney (27) and chicken embryo (20) enzymes. Unlike other dCMP deaminases, however, the yeast enzyme was highly specific for dCMP since halogenated (5-bromo-, 5-iodo-, 5-chloro-, 5-fluoro-) deoxycytidylates and 5-methyl-dCMP were found to be very poor substrates. Of these, only 5-fluoro-dCMP was deaminated at a substantial rate by the yeast enzyme. This finding is consistent with our observation that FdCMP is the only halogenated deoxycytidylate to exhibit toxicity to Tup^- strains, even though halogenated deoxyuridylates are generally toxic to these strains (4; L. Ross, personal communication).

Characterization of a dCMP deaminase-deficient mutant. Selection for resistance to FCdR has been used previously to isolate a dCMP deaminase-deficient mutant of *B. subtilis* (21). FCdR is only toxic to *S. cerevisiae* by virtue of catabolism to 5-fluorouracil, since media supplemented with uracil eliminates the toxic effect of this analog. The resistance of *S. cerevisiae* to the DNA-specific effect of FCdR reflects the apparent inability of this organism to phosphorylate deoxynucleosides. As an alternative selection procedure, we examined the effect of FdCMP in nucleotide-permeable (Tup^-) strains.

Preliminary studies demonstrated that FdCMP is toxic to Tup^- strains by virtue of its deamination to FdUMP, a potent inhibitor of the yeast thymidylate synthetase (2). Since dCMP deaminase contributes to cellular dUMP pools, the conversion of FdCMP to FdUMP via dCMP deaminase results in cell death. Thus, mutants deficient in this enzyme were expected to exhibit resistance to the toxic effect of FdCMP. The isolation of a dCMP deaminase-deficient mutant by this selection procedure was found to be complicated, however, by the appearance of numerous mutants that appeared to exhibit unusually high levels of nonspecific phosphatase activity. Although such mutants were unexpected, increased intracellular degradation of FdCMP to FCdR would account for the FdCMP-resistant phenotype if *S. cerevisiae* is unable to phosphorylate FCdR in vivo. To distinguish between these apparent phosphatase-overproducing mutants, *tup7* revertants, and true dCMP deaminase-deficient strains, FdCMP-resistant colonies were screened for cross-sensitivity to FdUMP. Phosphatase mutants and *tup7* revertants were expected to score as FdUMP resistant either because of enhanced intracellular degradation of FdUMP to 5-fluoro-2′-deoxyuridine, which is not toxic to *S. cerevisiae* (3), or because of the loss of the nucleotide permeability phenotype. Of 154 FdCMP-resistant colonies screened by this procedure, only 4 exhibited a marked sensitivity to FdUMP. Figure 3 illustrates the sensitivity of one of these strains (EMD1) to FdUMP, compared with that of the parental strain (B500C). Of these four FdUMP-sensitive isolates, only one was found to lack detectable dCMP deaminase activity in vitro.

Figure 4 shows the results of dCMP deaminase assays, using dialyzed crude lysates of both the parental (B500C) and mutant (EMD1) strains. The lack of dUMP formed in the mutant extract assay cannot be attributed to enhanced degradation of dUMP by a dUMP-specific phosphatase (33) since there was no corresponding increase in UV-absorbing material in the region of the chromatogram where bases and nucleosides elute (before dCMP). This was not the case for extracts of the apparent phosphatase-overproducing mutants which degraded both substrate (dCMP) and product (dUMP) to barely detectable levels when PP_i was omitted from the reaction buffer. Thus, the FdCMP-resistant, FdUMP-sensitive phenotype exhibited by the mutant strain EMD1 resulted solely from a deficiency of dCMP deaminase activity. We designated the allele responsible for this phenotype *dcd1*.

Figure 5 shows the growth rates of B500C and EMD1 in

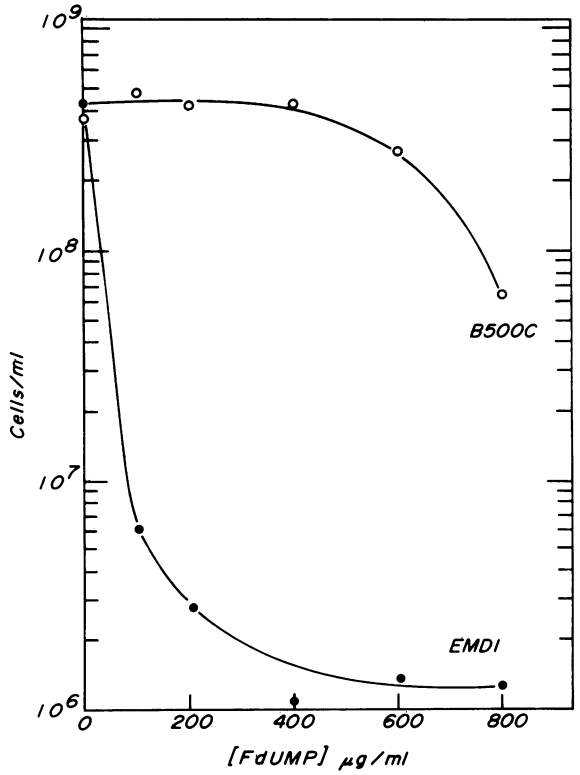

FIG. 3. Cell density after growth in YPD medium supplemented with various concentrations of FdUMP. Cultures were inoculated to an initial density of 2×10^6 cells per ml and grown for 48 h at 30°C.

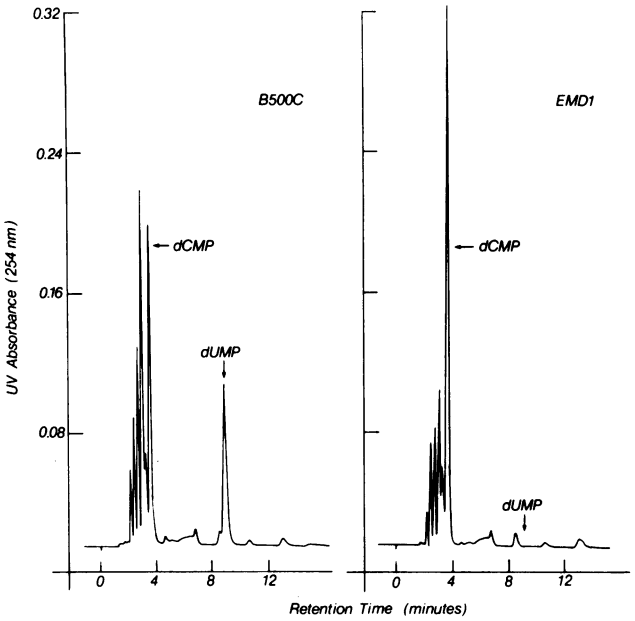

FIG. 4. High-pressure liquid chromatography analysis of dCMP deaminase activity from strains B500C (*MATα tup7-1*) and EMD1 (*MATα dcd1 tup7-1*).

both the presence and absence of FdCMP. From this graph, it can be seen that the mutant exhibited complete resistance to concentrations of FdCMP sufficient to block totally the growth of the parental strain. Previous studies of FCdR toxicity in mammalian cells have suggested that incorporation of FdCMP into DNA may be at least partially responsible for its killing effect (31). Since the growth rate of the dCMP deaminase-deficient mutant described here is the same in medium with or without FdCMP, the toxicity of this analog in *S. cerevisiae* likely results solely from its conversion to FdUMP.

The growth rate of the mutant in YPD medium alone was slightly reduced from that of the parental strain. This curious property was not caused by inhibition of intracellular dTMP synthesis since including dTMP in the medium did not restore growth of the mutant to the parental rate (Fig. 5). This feature did, however, appear to be a property associated with the enzyme deficiency since, in numerous crosses with a variety of strains, *dcd1* haploids were always recovered from among the slowest growing colonies.

Isolation and characterization of a dUMP auxotroph. In most organisms, dUMP is generated de novo from the deamination of dCMP or through the reduction of UDP via a series of reactions catalyzed by ribonucleotide reductase, nucleoside diphosphate kinase, and dUTP pyrophosphatase (Fig. 1). Since the *dcd1* allele blocked one of these two pathways, a combination of this marker with a second mutation affecting the UDP reduction pathway was expected to result in a dUMP auxotroph phenotype. Such an auxotroph was isolated after ethyl methanesulfonate mutagenesis of the dCMP deaminase-deficient strain EMD1. Table 1 illustrates the growth rate of this mutant (DRH1) in the presence of various concentrations of dUMP and dTMP. From these data, it can be seen that DRH1 exhibited an auxotrophic requirement for dUMP which could be satisfied also by exogenous dTMP, but not by deoxyuridine.

To determine whether the dUMP auxotrophic phenotype resulted from a combination of the *dcd1* allele and a second independent nuclear marker, meiotic segregation of the phenotype from a heterozygous diploid was examined. If the dUMP auxotrophy is a consequence of a mutation at a single locus, independent of the *dcd1* allele, then only 2:2 segregation of the phenotype is expected to occur. Of 47 tetrads analyzed, only 5 exhibited 2:2 segregation, whereas 34 exhibited 1:3 segregation, and 8 were 0:4 (auxotrophs:prototrophs). These results show that the dUMP auxotroph phenotype must result from a combination of the *dcd1* allele and a second mutation at an independent locus. The approximate 1:4:1 ratio of the 2:2, 1:3, and 0:4 segregations is indicative of two unlinked nuclear genes controlling a single property. The allele responsible for the dUMP auxotrophic requirement in the *dcd1* background was designated *dmp1*.

The reversion of the dUMP auxotrophic phenotype in DRH1 was low ($<5 \times 10^{-6}$). Of several phenotypic revertants isolated, none was found to exhibit detectable dCMP deaminase activity. This indicates that the *dcd1* allele is very stable. The *dmp1* marker by itself conveyed no readily discernible phenotype other than a slight swelling and distortion of cell shape.

DISCUSSION

In this paper we have reported initial characterization of dCMP deaminase from *S. cerevisiae* and the isolation of a

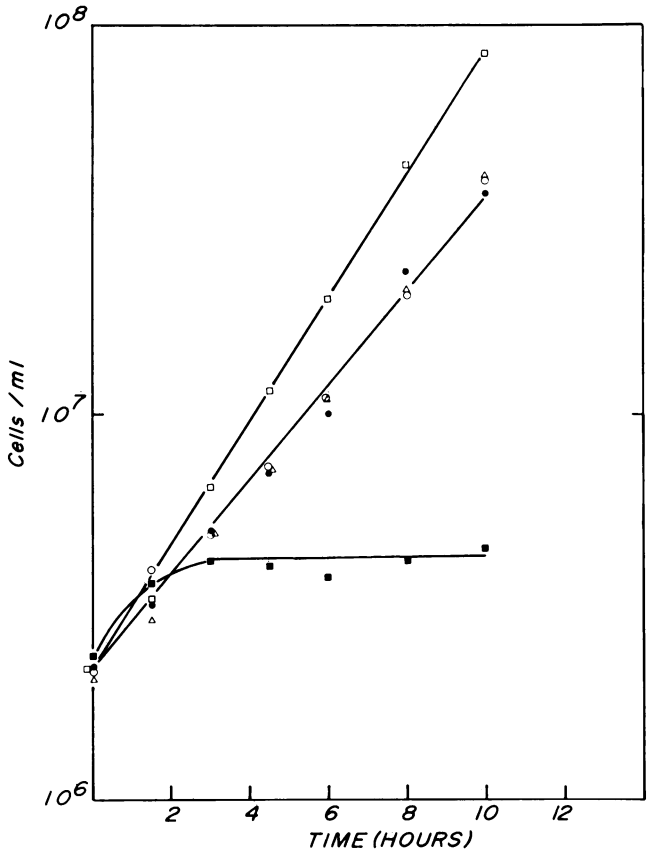

FIG. 5. Growth rates of strains B500C and EMD1 in the presence and absence of FdCMP. Symbols: □, B500C in YPD; ■, B500C in YPD plus 4 mg of FdCMP per ml and 120 μg of uracil per ml; ○, EMD1 in YPD; ●, EMD1 in YPD plus 4 mg of FdCMP per ml and 120 μg of uracil per ml; △, EMD1 in YPD plus 100 μg of dTMP per ml.

TABLE 1. Growth rates of DRH1 (*MATαdcd1 dmp1 tup7-1*) in YPD medium alone and supplemented with UdR, dUMP, and dTMP[a]

Supplement	Concentration (μg/ml)	Generation time (h)
None		>6.0
UdR	1,000	>6.0
dUMP	50	3.6
	100	2.7
	200	2.6
	400	2.6
dTMP	50	3.6
	100	3.9
	200	2.8
	400	2.5

[a] Strain DRH1 was grown to mid-log phase in YPD medium supplemented with dUMP (400 μg/ml). Cells were harvested, washed, and resuspended in medium with various concentrations of the indicated supplement. Call density at various time points was determined with a Coulter Counter, and generation times were calculated from these data.

mutant strain deficient in this activity. The yeast enzyme was found to exhibit properties very similar to those of dCMP deaminases isolated from higher eucaryotes, at least in terms of apparent K_m, activation by dCTP, divalent cation requirement, and pH optimum. A dCMP deaminase-deficient mutant was isolated in a *tup7* background by selection for resistance to FdCMP, followed by screening for cross sensitivity to FdUMP. The mutation responsible for this enzyme deficiency was designated *dcd1*.

The unusual sensitivity to FdUMP conferred by the *dcd1* allele presumably resulted from decreased intracellular synthesis of dUMP. In *B. subtilis* (21) and Chinese hamster fibroblasts (26), dCMP deamination supplies ca. 45 to 55% of the cellular dUMP pool. In Novikoff hepatoma cells, this contribution is substantially greater (14). Blockage of the dCMP deaminase pathway in yeast cells likely reduces the cellular level of dUMP sufficiently to allow FdUMP to compete more effectively for binding to thymidylate synthetase, but not severely enough to restrict DNA replication and normal cell growth.

Although the *dcd1* allele appeared to influence generation rate, this was not caused by limitation for dUMP or dTMP. It is possible that the yeast dCMP deaminase interacts physically with other enzymes involved in deoxynucleotide metabolism, as has been demonstrated previously for the T4-phage-encoded enzyme (7). Thus, some properties associated with the *dcd1* allele, such as reduced growth rate, may result from changes in physical interactions between the yeast dCMP deaminase and other enzymes involved in deoxynucleotide synthesis rather than blockage of the dCMP deamination reaction.

The isolation of the *dcd1* allele afforded us the opportunity to select for an additional, novel mutant of *S. cerevisiae* altered in another enzyme of deoxynucleotide metabolism. We assumed that a combination of the *dcd1* lesion and a second mutation, affecting an enzyme involved in the reduction of UDP to dUMP, would result in a strain exhibiting at least a partial auxotrophic requirement for dUMP. Such a mutant was recovered after ethyl methanesulfonate mutagenesis of a *tup7* strain harboring the *dcd1* allele.

Meiotic segregation of the dUMP auxotrophic phenotype from a heterozygous diploid revealed it to result from a combination of the *dcd1* allele and a second, independent mutation which we designated *dmp1*. The results of tetrad analysis, however, do not preclude the possibility of the *tup7* allele contributing to this dUMP auxotrophic phenotype. The *tup7* mutation, originally described by Bisson and Thorner (5), is allelic to *PHO80* and induces constitutive expression of phosphate-repressible acid and alkaline phosphatases (3). Since *tup7* mutants exhibit abnormally high levels of phosphatase activity, low cellular levels of dUMP, induced by a combination of the *dcd1* and *dmp1* mutations, may be reduced even further, to limiting amounts, by elevated cellular levels of phosphatase activity.

Although genetic analysis of the *dcd1* and *dmp1* markers suggests that either dCMP deamination or UDP reduction supplies sufficient dUMP to sustain growth of *S. cerevisiae*, we have not determined quantitatively the relative importance of each of these pathways in *S. cerevisiae*. However, since strains carrying the *dmp1* allele exhibit a slightly swollen "dumbbell" morphology, which is characteristic of yeast cells delayed in S phase (16), it is likely that the UDP reductase pathway is the more important source of cellular dUMP.

The *dcd1* and *dmp1* mutations described here should be useful in furthering studies of the potential role of deoxynucleotide pool balance in genetic stability and change. In addition, they also provide a selection system suitable for screening yeast genomic libraries for the dCMP deaminase gene. The thymidylate synthetase gene from *S. cerevisiae* was previously isolated in our laboratory by genetic complementation of a thymidylate synthetase (*tmp1*)-deficient mutant (32). Since *dcd1 dmp1 tup7* haploids exhibit a phenotype analogous to that of *tmp1* mutants, it should be possible to select either the *DMP1* or *DCD1* genes from appropriate gene pools.

Like thymidylate synthetase (22) and several other enzymes involved in deoxynucleotide synthesis (8, 11, 12, 17–19), dCMP deaminase has been found to exhibit periodic fluctuations in activity during the eucaryotic cell cycle (13, 29). A comparative study of thymidylate synthetase and dCMP deaminase gene expression in *S. cerevisiae* may reveal critical features of cell cycle-dependent gene regulation in this eucaryote.

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