Physiological and Enzymatic Properties of a Thymidine-Requiring *Pediococcus cerevisiae* Mutant

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We describe the isolation and characterization of a *Pediococcus cerevisiae* thymidine-requiring mutant and its thymidine-independent revertant. The mutant strain lacked thymidylate synthetase activity and had an absolute requirement for low concentrations (2 μg/ml) of thymidine in addition to a requirement for N-5-formyl tetrahydrofolic acid (folinate). Even at high concentrations (up to 500 μg/ml), thymine could not replace thymidine. In contrast to its wild-type parent, which grows only on folinate, the thymidine-requiring mutant (Thy− Fol+) was able to take up and grow on picogram quantities of unreduced folic acid. When both strains were grown on folinate, the Thy− Fol+ strain was at least 10²-fold more resistant to the folic acid analogs aminopterin and methotrexate than the wild-type strain. On the other hand, when grown on folic acid, the Thy− Fol+ strain was as sensitive to the folic acid analogs as the Thy+ Fol+ strain and was 10²-fold more sensitive than the wild-type strain grown on folinate. The thymidine-independent revertant (Thy+ Fol+) regained the wild-type level of thymidylate synthetase activity, but maintained the ability to take up and grow on unreduced folic acid like its Thy− Fol+ parent.

Conversion of dUMP to dTMP is catalyzed by thymidylate synthetase (EC 2.1.1.8) and is mediated by 5,10-methylene tetrahydrofolate, which serves both as the carrier of the single carbon unit and as a reductant; the dihydrofolate acid thus formed is reduced to tetrahydrofolic acid by dihydrofolate reductase (EC 1.5.1.3).

*Pediococcus cerevisiae* (formerly known as *Leuconostoc citrovorum*) strain 8081 (6, 11) is characterized by a unique requirement for N-5-formyl tetrahydrofolate (also known as folinate [20]), which is of pivotal importance for thymidylate synthesis, as well as for other C₁ transfer reactions. Unless supplied in high concentrations (microgram quantities), unreduced folate cannot substitute for folinate because *P. cerevisiae* (folate uptake negative [Fol−]) cannot take it up (14).

Aminopterin, a folic acid analog, indirectly prevents thymidylate biosynthesis by inhibiting dihydrofolate reductase. Aminopterin has been used to select thymidine-requiring mutants from several bacterial species (5, 18, 19, 22); we similarly isolated such a mutant from *P. cerevisiae*. This mutant strain is thymidylate synthetase negative (Thy−) and Fol+. We also isolated a thymidine-independent Thy+ Fol+ revertant. In this paper we describe some enzymatic and physiological properties of these mutants.

MATERIALS AND METHODS

**Bacteria and culture medium.** *P. cerevisiae* ATCC 8081 was maintained at 4°C in stabs of Micro Assay Culture Agar (Difco Laboratories, Detroit, Mich.). Cultures were grown in a chemically semidefined medium (8) in optically matched Kimax tubes (150 by 18 mm) containing 5 ml of medium. Folinate (folinic acid, Sigma Chemical Co., St. Louis, Mo.) was added aseptically to the medium after being autoclaved for 7 min at 15 lb/in².

Cultures were incubated for 24 or 48 h at 37°C. A Spectronic 88 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) was used to determine optical density at 600 nm.

**Isolation of mutants.** Thymidine auxotrophs were selected by the method of Okada et al. (18, 19) after UV irradiation for 55 s (to increase the mutation level) and two serial passages in semidefined liquid medium containing 200 pg of folinate per ml, 400 μg of thymidine per ml, and 400 μg of aminopterin per ml (to select for mutants). Thymidine-nonrequiring revertants were isolated after UV mutagenesis. We tested about 100 Thy− Fol+ mutants and about 100 Thy+ Fol+ revertants, and we randomly selected one of each to characterize further.

**Preparation of cell extracts.** Cultures for enzymatic determinations were grown in 1-liter Erlenmeyer flasks containing 600 ml of medium. The cultures were incubated without shaking for 24 h at 37°C. Cells were harvested and washed once with 0.05 M Tris buffer (pH 7.5) by centrifugation at 12,000 × g for 5 min at
4°C. The cells were concentrated 100-fold in the same buffer and disrupted by shaking for 45 s in the cold with small glass beads (diameter, 0.1 mm; 10 g of beads per 10 ml of suspension) in a disintegrator (type 2876; B. Braun Apparatebau Melsungen AG). After centrifugation for 30 min at 4°C and 16,000 × g, the supernatant obtained was used as the enzyme source.

Enzyme assays. Thymidylate synthetase was assayed spectrophotometrically by following the increase in optical density at 340 nm due to the conversion of 5,10-methylene tetrahydrofolate to dihydrofolate during the synthesis of thymidylate from deoxyuridylate (Δε = 6,400 M⁻¹ cm⁻¹) (23).

Dihydrofolate reductase was assayed by the method of Blakley and McDougall (2) by following the decrease in optical density at 340 nm caused by the reduction of dihydrofolate to tetrahydrofolate in the presence of the enzyme and NADPH. Both enzymatic determinations were made with a Varian Techtron UV-visible spectrophotometer (model 635).

Measurement of folic acid uptake. Bacteria were grown for 24 h at 37°C, harvested by centrifugation at 12,000 × g for 5 min at 4°C, washed twice in 0.85% NaCl (saline), and suspended in saline at a concentration of 2 mg (dry weight) of cells per ml. The uptake mixture consisted of 50 mM sodium phosphate buffer (pH 5.8) containing 1% glucose and [2-¹⁴C]folic acid (potassium salt; 50 mCi/mmol; Radiochemical Centre, Amersham, England). Cells at a concentration of 1 mg (dry weight) per ml were preincubated in this mixture without label in a shaking bath for 5 min at 37°C, and then ¹⁴C-labeled folic acid was added to a final concentration of 2 μM. At different times 1-ml samples were removed, filtered through membrane filters (pore size, 0.45 μm; diameter, 24 mm), and washed twice with 10 ml of cold saline. The filters were then transferred to counting vials and dried in a 65°C oven. To each vial was added 4 ml of scintillation fluid containing (per liter of toluene) 0.1 g of POPOP (1,4-bis[2]-(5-phenyloxazolyl)benzene) and 3 g of PPO (2,5-diphenyloxazolylbenzene). Radioactivity was determined with a Tri-Carb liquid scintillation spectrometer (model 3310; Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Growth of P. cerevisiae strains with varying thymidine concentrations. Our thymidine-requiring mutant did not grow in the chemically semidefined medium supplemented with 200 pg of folinate per ml; however, the addition of 2 μg of thymidine per ml permitted maximal growth (Fig. 1). Like the wild type, the Thy⁺ Fol⁺ revertant grew well in the absence of thymidine, and, as previously reported (7), the addition of thymidine enhanced the growth of both the wild type and the Thy⁺ Fol⁺ revertant (Fig. 1).

Effect of thymidine deprivation on the Thy⁻ Fol⁺ mutant. When a culture of the Thy⁻ Fol⁺ mutant was transferred from complete medium containing thymidine to the same medium deficient in thymidine, thymineless death of the mutant occurred (Table 1). After such a transfer, the cell viability (measured as the ability to form colonies on agar plates) decreased 10³-fold within 24 h when the cells were incubated at 37°C. Under the same conditions, the viable count of the wild-type strain increased 10³-fold.

Specificity of the thymidine requirement. Since thymidine could not be replaced by even 500 μg of thymine per ml, by 1,000 μg of vitamin B₁₂ per ml, or by 100 μg of deoxyribose per ml, we concluded that the requirement of the Thy⁻ Fol⁺ mutant for thymidine is specific.

Thymidylate synthetase and dihydrofolate reductase activities. Crude extracts of the Thy⁻ Fol⁺ and Thy⁺ Fol⁺ strains both contained dihydrofolate reductase activity like the activity of wild-type extracts (Table 2).

Whereas crude extracts of the wild-type cells

TABLE 1. Effect of thymidine deprivation on the Thy⁻ Fol⁺ mutant and the wild-type strain*

<table>
<thead>
<tr>
<th>Supplement(s)</th>
<th>Wild-type strain</th>
<th>Thy⁻ Fol⁺ mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD₆₅₀</td>
<td>Viable count</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.01</td>
<td>3.0 × 10⁵</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.55</td>
<td>4.5 × 10⁵</td>
</tr>
<tr>
<td>Folic acid +</td>
<td>0.76</td>
<td>3.5 × 10⁶</td>
</tr>
<tr>
<td>thymidine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cells were transferred from the semidefined medium (see text) containing 200 pg of folinate per ml and 2 μg of thymidine per ml to the same medium supplemented with 200 pg of folinate per ml and 2 μg of thymidine per ml. The optical density at 600 nm (OD₆₅₀) and the viable counts were determined after 24 h of incubation at 37°C. The viable count value at zero time was 10⁵ cells per ml of culture.
TABLE 2. Thymidylate synthetase and dihydrofolate reductase activities

<table>
<thead>
<tr>
<th>Strain</th>
<th>Thymidylate synthetase (nmol/mg of protein per h)</th>
<th>Dihydrofolate reductase (U/mg of protein per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Thy- Fol+</td>
<td>&lt;0.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Thy+ Fol+</td>
<td>2.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*The preparation of extracts and the enzyme assays were done as described in the text; 1 U of dihydrofolate reductase activity was defined as the amount of enzyme causing a 0.01-unit decrease in the optical density at 340 nm in 1 min.

Fol+ grown (see text)

Growth of the Thy- Fol+ revertant and the Thy+ Fol+ revertant on unred folic acid. Wild-type *P. cerevisiae* requires folic acid as a source of reduced folic acid. The wild-type, Thy- Fol+, and Thy+ Fol+ strains all had the same growth pattern on folic acid; 80 pg of folic acid per ml permitted 80 to 100% of the maximal growth of all three strains (data not shown). Unlike the parent wild-type strain, the Thy- Fol+ mutant also grew in medium supplemented with picogram quantities of unred folic acid. The Thy+ Fol+ revertant, which was similar to the wild type in its ability to grow without thymidine, retained the ability of the Thy- Fol+ mutant to grow on picogram quantities of folic acid (Fig. 2). Another Fol+ mutant, which was described previously as a pteroyl-glutamic acid (pte-glu) strain (15), was directly selected for folic use and showed a similar growth pattern on folic acid (Fig. 2).

Uptake of [14C]folic acid. Unlike the wild type, the Thy- Fol+ mutant, the Thy+ Fol+ revertant, and the Fol+ strain all took up [14C]folic acid (Fig. 3). We found that after incubation for 5 min at 37°C, the accumulation of labeled folic acid was 1,000 nmol/ml in the Thy- Fol+ strain, whereas it was only 200 nmol/ml in the Thy+ Fol+ strain; the accumulation in the Fol+ strain was 400 nmol/ml. We are presently investigating these quantitative differences.

Sensitivity to folic acid analogs. The Thy+ Fol+ mutant reacted differently than the wild-type strain to the folic analogs aminopterin and methotrexate. When grown on folic in the presence of increasing concentrations of methotrexate for 24 h, the Thy- Fol+ mutant was at least 103-fold more resistant to methotrexate than the wild-type strain. The Thy- Fol+ mutant was routinely grown with 2 µg of thymidine per ml (Fig. 4A). The addition of the same concentration of thymidine to a wild-type strain culture increased resistance to methotrexate fivefold (Fig. 4A). However, when the incubation period with the folic acid analog was doubled to 48 h, the wild-type strain grown in the presence of thymidine was as resistant to methotrexate as...

FIG. 2. Growth of the wild type, the Thy- Fol+ mutant, the Thy+ Fol+ revertant, and the Fol+ strain on different unred folic acid concentrations. Cells were grown for 48 h at 37°C in semidefined medium (see text) in the presence of 2 µg of thymidine per ml. Symbols: △, wild type; ○, Thy- Fol+ mutant; ▲, Thy+ Fol+ revertant; O, Fol+ strain. O.D., Optical density.

FIG. 3. Kinetics of folic acid uptake. The preparation of cells and the uptake procedure were as described in the text; 2 µM [14C]folic acid (FA) was used. Symbols: △, wild type; ○, Thy- Fol+ mutant; ▲, Thy+ Fol+ revertant; O, Fol+ strain.
FIG. 4. Effect of methotrexate on the wild type and the Thy− Fol+ mutant grown on folinate with or without thymidine. (A) Cells were grown in semidefined medium (see text) in the presence of 200 pg of folinate per ml and in the absence or in the presence of 2 μg of thymidine per ml for 24 h at 37° C. Methotrexate (MTX) was added at zero time. (B) Cells were grown as described above but for 48 h. Symbols: △, wild type without thymidine; ▲, wild type with thymidine; ○, Thy− Fol+ mutant with thymidine. O.D., Optical density.

the Thy− Fol+ strain grown under the same conditions (Fig. 4B). After 24 h of incubation in the presence of 10−4 M methotrexate and 2 μg of thymidine per ml there was no visible growth of the wild-type strain, whereas the viable count was 2 × 10^6 cells per ml, a 20-fold increase compared with the inoculum (Table 3). Prolongation of the incubation time of the culture to 48 h resulted in the appearance of visible growth (optical density at 600 nm, 0.55), and the viable

### TABLE 3. Effect of methotrexate on the viable counts of the *P. cerevisiae* wild-type strain and the Thy− Fol+ mutant in the absence and presence of thymidine

<table>
<thead>
<tr>
<th>Supplement(s)</th>
<th>Wild-type strain</th>
<th>Thy− Fol+ mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>OD_600</td>
<td>Viable count (cells/ml of culture)</td>
</tr>
<tr>
<td>Folinate</td>
<td>0.48</td>
<td>4.2 × 10⁷</td>
</tr>
<tr>
<td>Folinate + thymidine</td>
<td>0.65</td>
<td>2 × 10⁸</td>
</tr>
<tr>
<td>Folinate + methotrexate (10−⁶ M)</td>
<td>0.01</td>
<td>6 × 10⁴</td>
</tr>
<tr>
<td>Folinate + methotrexate (10−⁷ M)</td>
<td>0.01</td>
<td>5.5 × 10⁴</td>
</tr>
<tr>
<td>Folinate + thymidine + methotrexate (10−⁴ M)</td>
<td>0.01</td>
<td>2 × 10⁹</td>
</tr>
<tr>
<td>Folinate + thymidine + methotrexate (10−⁶ M)</td>
<td>0.40</td>
<td>2.5 × 10⁷</td>
</tr>
<tr>
<td>Folinate + thymidine + methotrexate (10−⁷ M)</td>
<td>0.40</td>
<td>2.5 × 10⁷</td>
</tr>
</tbody>
</table>

*Cells were grown in semidefined medium (see text) supplemented with 200 pg of folinate per ml, 2 μg of thymidine per ml, and methotrexate, as indicated. The optical density at 600 nm (OD_600) and the viable counts were determined after 24 and 48 h of incubation at 37° C. The viable count value at zero time was 10⁵ cells per ml of culture for both strains.*
FIG. 5. Effect of methotrexate on the Thy− Fol+, Thy+ Fol+, and Fol+ strains grown on folic acid and thymidine. Cells were grown in semidefined medium (see text) in the presence of 200 μg of folic acid per ml and 2 μg of thymidine per ml for 48 h at 37°C. Methotrexate (MTX) was added at zero time. Line A, Thy− Fol+ mutant; line B, Thy+ Fol+ revertant; line C, Fol+ strain. O.D., Optical density.

count increased 100-fold compared with the count at 24 h. Thus, the growth observed was due to cell division. In the absence of thymidine, methotrexate caused the death of the wild-type strain; there was a sharp decrease in the viable count to <10^6 cells per ml after 48 h of incubation, presumably due to thymineless death by inhibition of thymidylate synthesis. Similar results were obtained when aminopterin was used in place of methotrexate (data not shown).

The Thy+ Fol+ mutant and the Fol+ strain were equally sensitive to methotrexate whether they were grown on folic acid (Fig. 5) or on folinate (data not shown); however, when the Thy− Fol+ mutant was grown on folic acid, its sensitivity to methotrexate increased at least 10^2-fold (Fig. 5 and 4B).

Properties of the P. cerevisiae wild-type strain, the Fol+ strain, the Thy− Fol+ mutant, and the Thy+ Fol+ revertant. Table 4 shows the properties of the P. cerevisiae wild-type strain, the Fol+ strain, the Thy− Fol+ mutant, and the Thy+ Fol+ revertant.

DISCUSSION

We selected a Thy− Fol+ mutant from a UV-irradiated culture of P. cerevisiae by growing the cells in the presence of high concentrations of thymidine (400 μg/ml) and the folate analog aminopterin (400 μg/ml). When this method was used, the culture was selectively enriched for mutants with the following characteristics: (i) an absolute requirement for exogenously supplied thymidine due to the loss of thymidylate synthetase activity (deprivation of thymidine causes thymineless death); (ii) a new ability to take up and grow on picogram quantities of unredrred folic acid in place of folinate; and (iii) high resistance to the folate analogs aminopterin and methotrexate. The Fol+ strain (the pte-glu strain of Mandelbaum-Shavit and Grossowicz [15]), which was isolated in the absence of any folate analog, and the Thy+ Fol+ revertant, which was exposed to a folate analog in its history (when its Thy− Fol+ parent was isolated), were much more sensitive to aminopterin and methotrexate than the Thy− Fol+ mutant.

### TABLE 4. Properties of P. cerevisiae strainsa

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Growth without thymidine</th>
<th>Thymidylate synthetase</th>
<th>Uptake of unred folate</th>
<th>Sensitivity to methotrexateb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Grown on folinate for:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Wild type Mutant</td>
<td>Thy+ Fol−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>10^-7</td>
</tr>
<tr>
<td></td>
<td>Thy− Fol+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Revertant</td>
<td>Thy+ Fol+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5 x 10^-9</td>
</tr>
<tr>
<td>pte-glu mutant</td>
<td>(Thy+) Fol+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5 x 10^-9</td>
</tr>
</tbody>
</table>

a UV mutagenesis and the selection procedure for the Thy− Fol+ mutant and the Thy+ Fol+ revertant are described in the text. The Fol+ strain was obtained by direct selection, as described by Mandelbaum-Shavit and Grossowicz (15).
b Expressed as the methotrexate concentration (molar) which produced an optical density at 600 nm of 0.
Bertino and Stacey (1) showed that mutation to thymine dependence entails increased resistance to the folate analogs aminopterin and trimethoprim. By binding very strongly to the enzyme dihydrofolate reductase, these analogs prevent the generation of tetrahydrofolate, which is an important cofactor in \( C_1 \) transfer reactions and is used mainly for thymidylate synthesis. Therefore, after prolonged incubation in the presence of high concentrations of aminopterin most of the survivors lack the enzyme thymidylate synthetase (1). In the absence of thymidylate synthetase, tetrahydrofolate is not oxidized, and the need for dihydrofolate reductase activity is bypassed. Thus, the Thy\(^-\) Fol\(^+\) mutant requires exogenously supplied thymidine, and it follows that this mutant should be resistant to aminopterin and methotrexate. It is interesting that after 48 h of incubation in the presence of the folate analog and thymidine, the wild-type strain became as resistant to methotrexate as the Thy\(^-\) Fol\(^+\) mutant. As Table 3 shows, growth was due to cell division and not just filamentation.

In **Bacillus subtilis** resistance to folate analogs is caused by the specific antifolate resistance mutation *afo* (4). It is possible that the Thy\(^-\) Fol\(^+\) mutant carries a similar additional mutation for antifolate resistance, but the fact that when the Thy\(^-\) Fol\(^+\) mutant is grown on folic acid, it is as sensitive to methotrexate as the Thy\(^+\) Fol\(^+\) revertant and the Fol\(^+\) strain (Fig. 5) argues against this possibility.

When grown on folinate, the Thy\(^+\) Fol\(^+\) revertant and the Fol\(^+\) strain are 100 times more sensitive to aminopterin and methotrexate than the wild-type strain (data not shown). This increased sensitivity may be explained by the increase in folate uptake, which presumably is associated with increased folate analog uptake in these strains, as suggested by Bertino and Stacey (1) and Farmer (4). Such an increase in the uptake of folate and its analogs presumably occurs in the Thy\(^-\) Fol\(^+\) strain, but this strain is still less sensitive to aminopterin and methotrexate when it is grown on folinate than when it is grown on folate; it follows that the absence of thymidylate synthetase activity is more important than increased folate uptake in determining sensitivity to folate analogs.

Our data strongly suggest that our Thy\(^-\) Fol\(^+\) mutant carries at least two separate mutations; it is thymidineless (Thy\(^-\)) and is able to take up and multiply on picogram quantities of unreduced folate (Fol\(^+\)). Although unlikely, the possibility that this strain may even carry a third mutation for antifolate resistance cannot be completely eliminated.

The existence of thymine-requiring mutants shows that exogenous thymine can be used for thymidylate synthesis and that the internal deoxyribosyl groups are furnished by increased catabolism of deoxyribonucleotides by the crucial enzyme thymidine phosphorylase (12, 17, 21). The *P. cerevisiae* Thy\(^-\) Fol\(^+\) mutant could use only thymidine; even at a concentration of 500 \( \mu \)g/ml, thymine could not support growth. On the other hand, very low concentrations of thymidine (2 \( \mu \)g/ml) were sufficient to support optimal growth of this strain. Thymine-requiring mutants of *Escherichia coli* (19) and *Bacillus megaterium* (22), which were also selected in the presence of aminopterin, require much higher concentrations of thymine (20 to 25 \( \mu \)g/ml) to sustain growth. From such strains, low-thymine-requiring mutants, which are able to grow on 1 to 2 \( \mu \)g of thymine per ml, can be derived as a result of a second mutation (3, 16, 17). The lactobacilli, including the genus *Pediococcus*, differ from most other bacteria in deoxyribonucleotide metabolism (13, 24). We have found no thymidine phosphorylase activity in *P. cerevisiae* strains (data not shown), which may explain the high specificity of the thymidine requirement of the Thy\(^-\) Fol\(^+\) mutant.

*P. cerevisiae* strains differ from *E. coli* and *Bacillus* in their requirement for an exogenous supply of folates (reduced) (6, 11). The fact that Fol\(^+\) appears simultaneously with Thy\(^-\) does not seem to be accidental and is being investigated by us, as the availability of partly reduced folate (dihydrofolate) may aid in overcoming inhibition by folate analogs.

It was found recently that in human beings (9) and in some in vitro cell lines (10), thymidine affords better protection than folinate (leucovorin rescue) against the cytotoxic effect of methotrexate. Although a procaryote, *P. cerevisiae* depends on exogenous folic acid, like humans and other mammals. Our studies with *P. cerevisiae* mutants may serve as an interesting and useful model for studying folate-antifolate interactions.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**

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