Isolation and Characterization of Mutants of *Saccharomyces cerevisiae* Auxotrophic and Conditionally Auxotrophic for 5′-dTMP

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An improved method for isolation of yeast mutants auxotrophic for 5′-dTMP is presented. The procedure employs the two folic acid antagonists aminopterin and sulfanilamide (SAA). Selectiveness of the procedure depends on concentration of SAA and time of incubation.

44 mutants auxotrophic and 3 conditionally auxotrophic for 5′-dTMP were isolated. All belong to one complementation group. The corresponding gene was designated *TMP1*. Tetrad dissection revealed its chromosomal nature. *TMP1* is not closely linked to the genes *ADE2*,, *LEU1*, *ARG4*, *ILV2*, *HIS5*, *LYS1* and the mating type locus. With the centromere-linked genes *ARG4* and *LEU1* gene *TMP1* exhibited second division segregation frequencies of 0.42 and 0.53 respectively, indicative of centromere-linkage.

Strains auxotrophic and conditionally auxotrophic for 5′-dTMP were all respiratory deficient *(petite)*. Genetical analysis indicates that the *petite* phenotype is due to loss of the rho factor in cells harbouring either *tmp1* or $tmp1^{ts}$ alleles.

Introduction

In a recent paper (Fäth, Brendel, Laskowski, and Lehmann-Brauns [1]) we reported a procedure for the isolation of mutants of *Saccharomyces cerevisiae* auxotrophic for 5′-dTMP. The selectiveness of this method was rather poor in that only one percent of the clones arising on the screening medium were true auxotrophs. Now we wish to present a procedure highly selective. In addition we give some genetical data concerning the 5′-dTMP auxotrophy.

Materials and Methods

Strains

Strain 211-1aMT2-1 *ilv2 typ1 tlr TMP1* of *S. cerevisiae* (= strain *typ1 TMP1* in the text): As cited in Fäth *et al.* [1]. Strain 211-1aMT2-1 *ilv2 typ1 tlr tmp1-1* of *S. cerevisiae* (= strain *typ1 tmp1-1* in the text): Same characteristics as strain *typ1 TMP1* and auxotrophic for 5′-dTMP. Strain 211-1aMT2 *ilv2 typ1 TMP1* of *S. cerevisiae:* As cited in Fäth *et al.* [1]. This strain was generally used for the isolation of all the other *typ1 tmp1* mutants. – The markers of the strains employed in crosses are given in Table I.

Table I. Genotype of strains used in crosses.

211-1aM	*a ilv2 typ1 tmp1-2*
211-1aM	*a ilv2 typ1 tmp1-4*
211-1aM	*a ilv2 typ1 tmp1-10^{ts}*
MB1001-3C	*a arg4-17 his5-2 ade2-1 lys1-1*
MB1001-1D	*a arg4-17 his5-2 ade2-1 lys1-1*
KC370	*a arg4-17 his5-2 ade2-1 lys1-1 leu1-12 rad2-16*

The original strain 211-1aM is described in Fäth and Brendel [2], strain KC370 by Resnick [3]. Strains with the prefix MB were synthesized by the first author.

Media

1. Medium N, medium I and medium R: See Fäth and Brendel [2] and Fäth *et al.* [1]. 2. Medium S: Medium N, plus 50 μg aminopterin (Serva)/ml, plus 15 μg $Na_2 \cdot 5'$-dTMP (Merck)/ml. 3. Minimal-glucose medium: Medium N without amino acids. – When testing for respiratory proficiency glucose was replaced by 3% glycerol (doubly distilled, Merck). Solid media contained 2% agar (Difco). When further components were added to the media this is given in parentheses in the text.

Culture conditions of cells

1. Improving the screening procedure of *tmp* mutants: Strain *typ1 TMP1* or strain *typ1 tmp1-1* were pre-grown in medium N (15 μg $Na_2 \cdot 5'$-dTMP/ml) at our standard conditions for 24 hours (Fäth and Brendel [2]; Fäth *et al.* [1]). The cells were, without washing, diluted in phosphate buffer (0.067 M, pH 7.0) and 200 each were plated onto the media described in Results. Plates were incubated at 30 °C.

Mutagenization

This was done with ethyl methane sulfonate (EMS) as described in Fäth *et al.* [1]. Mutagenized

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cells were incubated at 30 °C or 36 °C in medium N (30 μg Na_2·5′-dTMP/ml) for 4 hours and then plated onto medium S. During the 4 hours of incubation in the medium N (Na_2·5′-dTMP) no net growth did occur. Therefore we assume that all the *typ1 tmp* clones isolated from medium S stem from independent mutational events.

Genetical methods

Mating, isolation of zygotes, sporulation, and ascus dissection were performed in standard manner (Hawthorne and Mortimer [4]).

Results

The improved screening procedure

The rationale of the screening for 5′-dTMP auxotrophs (*typ1 tmp* mutants) has been explained in Fäth *et al.* [1]. The screening medium described there contained aminopterin (APT) as the sole inhibitor of folic acid metabolism. And this medium was reported to allow growth of some large colonies (putative *typ1 tmp* mutant clones) plus a lot of small ones when EMS-mutagenized *typ1* cells were plated on it. Most of the putative *typ1 tmp* mutant clones were found to be phenotypically *TMP* (Fäth *et al.* [1]), and so were the small clones (Fäth, unpublished data). Checking the isolated clones for growth on medium I (as defined in Fäth *et al.* [1]) revealed all of them being as sensitive to it as the corresponding *typ1 TMP* parent. From these findings we concluded that the APT-screening medium (medium S) does give a certain growth advantage to *typ1 tmp* mutants but that it obviously does not exclude colony forming of the *typ1 TMP* parent.

Thus, as an alternative to medium S, we tested the standard medium R (as defined by Fäth *et al*, [1]), but void of adenine, as a screening medium. This medium contains APT plus 4 – 6 mg sulfanilamide (SAA)/ml. 10^6 EMS-mutagenized cells of strain 211-1aMT2 each were plated onto 20 plates of this (APT + SAA)-screening medium. However, only two tiny colonies were found – even after a two weeks' incubation. Both of them could be identified as *typ1 tmp* clones. This finding suggested: By APT plus appropriate amounts of SAA (< 4 mg/ml) screening conditions might be obtained that do not allow visible growth of the *typ1 TMP* parent but permit near normal growth of a *typ1 tmp* mutant.

We examined this by parallely plating strain *typ1 tmp1-1* and strain *typ1 TMP1* onto a series each of standard medium S additionally supplemented with 0 – 500 μg SAA/ml (Materials and Methods). Figs 1 and 2 * demonstrate that, in the presence of

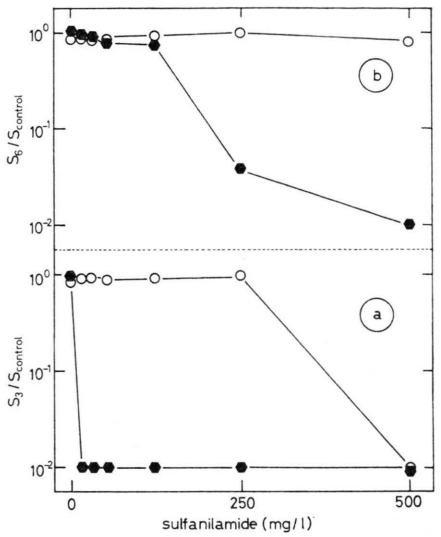

Fig. 1. Survival of strains auxotrophic and prototrophic for 5′-dTMP on medium S (SAA). a. Survival after a 3 days' incubation; b. survival after a 6 days' incubation, —●—, strain *typ1 TMP1*; —○—, strain *typ1 tmp-1*.

APT, SAA clearly enhances the selectiveness for the *typ1 tmp* mutant. And it is obvious from Fig. 1 a *vs* Fig. 1 b that the strength of (APT + SAA)-selectiveness depends on the time of incubation for SAA-concentrations between 0 and 500 μg SAA/ml: 20 μg SAA/ml are well sufficient to give excellent selectiveness after a 2 – 3 days' incubation (Fig. 1 a). But such an amount of SAA will not suffice when incubation is prolonged for 3 – 4 days. To obtain good selectiveness after a 6 days' incubation the SAA-concentration must be increased to 250 – 500 μg SAA/ml (Fig. 1 b). A medium S plus 20 μg SAA/ml, therefore, does not absolutely prevent colony forming of the wild type. The same is true for a medium S supplemented with SAA up to 250 μg/ml. This is already indicated by the fact that after a 3 days' incubation very tiny (non-countable) wild type colonies have appeared on medium S plus 20 – 250 μg SAA/ml. After the same time of incubation the *typ1 tmp1-1* colonies have already grown to near normal size. In contrast, no

* Fig. 2 see Table on page 736 a.

growth of wild type colonies was observed on a medium S plus 500 μg SAA/ml – even after a two weeks' incubation. However, after a 6 days' incubation on a medium S plus 500 μg SAA/ml the *typ1 tmp1-1* colonies are significantly reduced in size (Fig. 2, lower row).

Thus, optimal selective conditions can be obtained by appropriately varying the SAA-concentration and the time of incubation. As standard screening conditions a medium S (20 μg SAA/ml) and a 2 – 3 days' incubation will do good service. This may be seen from the results of the following experiment: With 200 cells of *typ1 tmp1-1* plus 10^6 cells of *typ1 TMP1* plated onto medium S plus 20 μg SAA/ml we found that after a 2 – 3 days' incubation all the normally sized colonies were *typ1 tmp1-1* clones. The 10^6 wild type cells exhibited but a hardly visible background growth.

Genetical characterization

47 mutants auxotrophic for 5′-dTMP (*tmp* mutants) were isolated from two batches of EMS-mutagenized cultures of strain 211-1aMT2. In one batch the cells were screened for *tmp* mutants at 30 °C, in the other at 36 °C. This higher temperature allowed the isolation of three conditional *tmp* mutants designed tmp^{ts}. They can grow without 5′-dTMP at 26 °C but not at 36 °C. All *tmp* mutants isolated so far are respiratory deficient *(petite)*. This is readily explained by their procedure of isolation in which APT and dimethyl sulfoxide are employed. Both chemicals are known to be inducers of the cytoplasmic *petite* mutation (Wintersberger and Hirsch [5, 6]; Yee, Tsuyumu and Adams [7]).

All *tmp* mutants have the same mating type *α*. The first two crosses with haploid *a*-maters prototrophic for 5′-dTMP were performed to obtain information on the segregation of the *tmp* marker and to get *tmp* mutants with the *a*-mating type for complementation studies. The results of these efforts are summarized in Table II, upper portion. Diploids heterozygous for the *tmp* marker sporulate normally but after tetrad dissection the *tmp* ascospores very seldom grow into a clone. The *tmp* marker proved to have such a negative effect on spore survival that none of the spores of diploid MB1050 and only five of the spores of diploid MB1051 exhibited auxotrophy for 5′-dTMP. Thus, nearly all of the tetrads dissected showed a segregation 2 *TMP:* 2 lethal. All other markers (Table I) segregated normally in the surviving spores, including the five *tmp* spores. The five *tmp* spores were *petite* while all *TMP* spores (all spores from the dissection of MB1050 and all but five from MB1051) gave rise to respiratory proficient *(grande)* strans. This indicates a correlation of the 5′-dTMP auxotrophy with respiratory deficiency as the handling of diploids MB1050 and MB1051 did not include the application of known *petite* inducing chemicals at any step during genetical analysis.

Of the five *tmp* strains obtained by tetrad dissection of MB1051 three had *a* and two *α* mating type. Complementation studies were carried out by mating strain MB1051-27C *(a arg4 lys1 leu1 tmp)* to all 47 isolated 5′-dTMP auxotrophs *(α ilv2 typ1 tmp)*. Mating was controlled by replica-plating onto minimal-glucose (30 μg Na_2·5′-dTMP/ml) where growth is due to complementation of standard

Table II. Viability of spores and segregation of the *TMP* marker from diploid strains.

Zygote	Sporulation [% Asci]	Asci dissected	Fractional viability of spore tetrads 1/4	2/4	3/4	4/4	Segregation of *TMP* marker
MB1050 *(TMP1 × tmp1-2)*	>50	34	16	18	0	0	All spores *TMP*.
MB1051 *(TMP1 × tmp1-4)*	31	79	16	59	4	0	2*TMP*:1*tmp* in the 4 tetrads with 3 spore viability; 1*TMP*:1*tmp* in 1 tetrad with 2 spore viability. All other spores *TMP*.
MB1067 *(TMP1 × tmp1-10^{ts})*	>50	51	0	3	18	30	2*TMP*:2*tmp^{ts}* in all 30 tetrads with 4 spore viability.
MB1072 *(TMP1 × tmp1-10^{ts})*	>50	61	0	24	23	14	2*TMP*:2*tmp^{ts}* in all 14 tetrads with 4 spore viability.
MB1074 *(TMP1 × tmp1-10^{ts})*	61	72	0	22	24	26	2*TMP*:2*tmp^{ts}* in all 26 tetrads with 4 spore viability.
MB1076 *(TMP1 × tmp1-10^{ts})*	64	76	0	9	18	49	2*TMP*:2*tmp^{ts}* in all 48 tetrads, 3*TMP*:1*tmp^{ts}* in one tetrad.

nutrional markers. Complementation of the *tmp* markers was monitored on minimal-glucose. All crosses showed complementation on minimal-glucose (5′-dTMP) and none on minimal-glucose. Therefore, all *tmp* mutants are considered to belong to one complementation group and are designated *tmp1-1* to *tmp1-47*, the three conditional *tmp* mutants bearing the allele numbers *tmp1-10^{ts}*, *tmp1-11^{ts}* and *tmp1-12^{ts}*.

The apparent lethality of the alleles *tmp1-2* and *tmp1-4* in tetrad dissections (Table II, upper portion) was partially overcome by the use of the alleles conferring conditional auxotrophy for 5′-dTMP. Table II, lower portion, shows the results of tetrad dissections of 4 diploids heterozygous for *tmp1-10^{ts}*. Survival of spores carrying the *tmp1-10^{ts}* allele is much higher than of spores carrying the *tmp1-4* allele. This is to be expected as thymidylate biosynthesis is still functional at the permissive temperature of 26 °C in spores harboring the *tmp1-10^{ts}* allele. Still, four spore survival at 26 °C is not very high and the tetrads with fractional spore viabilities of 3/4 or 2/4 are nearly always caused by the loss of spores containing the allele *tmp1-10^{ts}*. Complete tetrads clearly show a 2:2 segregation for the *tmp1* marker indicating its chromosomal localization. Thus mutation of gene *TMP1* leads to auxotrophy for 5′-dTMP in *S. cerevisiae*. Tetrad analysis also revealed the *TMP1* gene not to be linked to any of the other markers present in the crosses: There was no indication of linkage to *ade2*, *lys1*, *his5*, *leu1*, *arg4*, *ilv2* and the mating type locus. Centromere-linkage was tested employing the centromere-linked markers *leu1* and *arg4*. The results of the tetrad analysis are shown in Table III: *TMP1* exhibits a second division segregation frequency indicative of centromere-linkage.

Table III. Segregation of *tmp1* in relation to the centromere-linked genes *leu1* and *arg4* (pooled data from zygotes MB1067, MB1072, MB1074 and MB1076).

Gene Pair	PD	NPD	T	%T	SDS Frequency *
leu1—tmp1	11	17	34	55	0.53
arg4—tmp1	21	25	51	53	0.42

* Second division segregation frequency was determined according to Perkins [8] using the SDS frequencies 0.04 for *leu1* and 0.16 for *arg4* as given by Hawthorne and Mortimer [4].

The crosses employing the conditional *tmp1* marker *tmp1-10^{ts}* yielded an interesting result: All spores carrying the *tmp1-10^{ts}* allele gave rise to *petite* haploid strains, though germination was carried out at the permissive temperature and in the presence of 5′-dTMP (Table IV). In more than 900 spores tested *tmp1-4* and *tmp1-10^{ts}* were always associated with the *petite* phenotype whereas all 5′-dTMP prototrophic clones were *grande*.

There are two alternate explanations possible for these phenomena: 1. The isolation of a *tmp1* mutant is only possible when a closely linked *PET* gene has mutated as well. Thus a *tmp1* mutant is obtained which is also a segregational *petite*. 2. Any *tmp1* allele confers the *petite* character to the cell by eliminating tho *rho* factor (ϱ) during clonal growth.

To distinguish between these alternatives, 20 revertants from 5′-dTMP auxotrophy to prototrophy at 36 °C (*tmp1-10^{ts}* to *tmp1-10^{ts}+*) were isolated. They could grow without 5′-dTMP at 36 °C but were still *petite*. Three of these revertants,

Table IV. Linkage of 5′-dTMP auxotrophy with respiration deficiency (random spore segregation) *.

Zygote (cross)	Spores tested	Number of spores with phenotype *TMP grande*	*TMP petite*	*tmp grande*	*tmp petite*
MB1051 (*TMP1*ϱ^+ × *tmp1-4 petite*)	146	141	0	0	5
MB1067 (*TMP1*ϱ^+ × *tmp1-10^{ts} petite*)	180	102	0	0	78
MB1072 (*TMP1*ϱ^+ × *tmp1-10^{ts} petite*)	181	128	0	0	53
MB1074 (*TMP1*ϱ^+ × *tmp1-10^{ts} petite*)	176	95	0	0	81
MB1076 (*TMP1*ϱ^+ × *tmp1-10^{ts} petite*)	250	132	0	0	118

* Spores are from the complete and incomplete tetrads described in Table II.

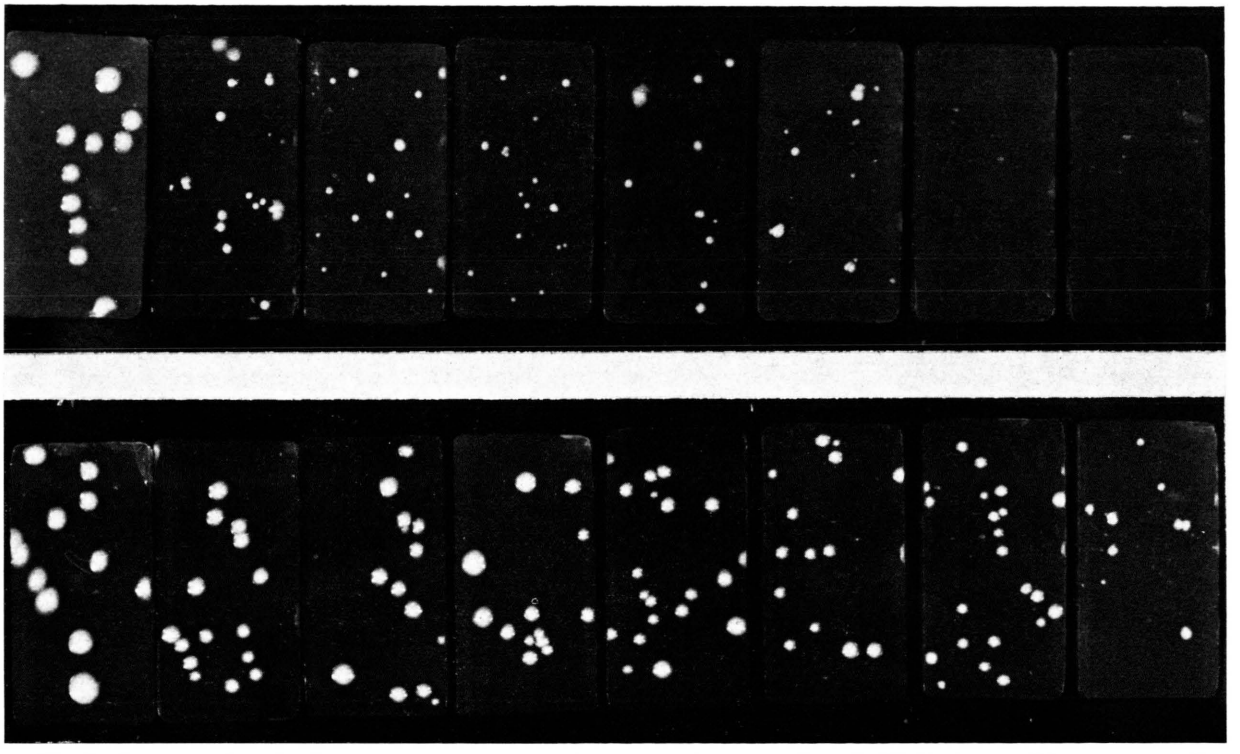

Fig. 2. Demonstration of colony appearence after a 6 days' incubation on medium S (SAA) (= conditions corresponding to those in Fig. 1 a). Upper row: Strain *typ1 TMP1*; lower row: Strain *typ1 tmp1−1*. From left to right: First agar slab, medium N (15 μg Na_2·5′-dTMP/ml); second to eighth agar slab, medium S with increasing amounts of SAA, the concentrations of which are given on the abszissa of Fig. 1.

tmp1-10^{ts}+1, *+2* and *+3*, were mated with haploids of opposite mating type and *TMP1*ϱ^+ genotype. Ascus dissection of these diploids yielded tetrads nearly all of which exhibited the 4 *TMP*ϱ^+ : 0 *tmp*ϱ^- segregational pattern typical for a diploid constructed by mating *grande* × cytoplasmic *petite* (Ephrussi, Hottinguer and Tavlitzki [9]) (Table V).

Table V. Segregation of *petite* phenotype from three zygotes constructed by mating wild type with three independent revertants to 5′-dTMP prototrophy.

Cross	Four spore tetrads *	4 TMPϱ^+: 0 tmpϱ^-	3 TMPϱ^+: 1 tmpϱ^-
a TMPϱ^+			
× *α tmp1-10^{ts}+1*	12	11	1
× *α tmp1-10^{ts}+2*	14	14	0
× *α tmp1-10^{ts}+3*	12	11	1

* 24 asci were dissected from each diploid.

Discussion

Our original concept of yeast *tmp* mutant isolation (Fäth *et al.* [1]) was based on two criteria: 1. The folic acid antagonist aminopterin (APT) should inhibit intracellular supply of tetrahydrofolic acid (THFA) for a *tmp* mutant and a wild type cell as well. 2. Omission of adenine from the APT-supplemented medium should compel both genotypes to perform normal purine nucleotide biosynthesis. With these conditions the intracellular THFA pool should be decreased onto an inhibitory level in the wild type cell. In contrast, a *tmp* mutant was expected to maintain a THFA pool-level well sufficient for growing up to a near normal sized clone on the selective medium.

As is demonstrated by Fig. 1 and Fig. 2 these conditions do not suffice to exclude colony forming of the wild type – though a significant growth advantage of the *tmp* mutant clones is manifest. This suggests the APT-inhibition of *dihydrofolic acid (DHFA): reductase* to be leaky enough as to permit THFA synthesis via this enzyme. And apparently the reduced output of THFA by *DHFA: reductase* is sufficient to more or less ensure the wild type cell's THFA requirement for normal-way purine nucleotide biosynthesis – despite the THFA consuming *thymidylate synthetase* present. In contrast, if together with APT the APT-synergist sulfanilamide (SAA) (Brown [10]) is employed in "high" concentrations, the wild type and the *tmp* mutant as well cannot form visible colonies on the selective medium. This suggests: "High" amounts of SAA too drastically reduce the supply of *DHFA: reductase* with *de novo* DHFA. As a consequence the enzyme's output of THFA will be too low to allow sufficient clonal growth even of the THFA non-consuming *tmp* mutant. Thus, if one wishes to obtain well visible *tmp* mutant clones on the selective medium and to simultaneously get mere background or sub-background growth of the wild type, one cannot enforce this by simply adding as much SAA as possible to the APT-screening medium. Supply of *de novo* DHFA for *DHFA: reductase* must still be permitted to a certain extent. And it is – within the limits deducible from Fig. 1 – left to the experimentator's choice how much of SAA should be employed, *i.e.* how much of *de novo* DHFA synthesis should be admitted. He may choose any SAA-concentration (>0 µg SAA/ml) given in Fig. 1. However, for a successful *tmp* mutant screening he simultaneously should take care of the time of incubation.

The *tmp1* alleles exhibit two interesting characteristics: 1. They apparently always confer the *petite* phenotype. This seems to be due to the loss of the rho factor in the absence of thymidylate biosynthesis (Table V). Even the *tmp^{ts}* alleles – at permissive temperature – lead to loss of respiration proficiency. The *TMP1* gene apparently codes for *thymidylate synthetase (ts)* as the *tmp1* and *tmp1^{ts}* alleles – at restrictive temperature – lead to an accumulation of 5′-dUMP in the growth medium (Majid, unpublished data). This suggests a minor malfunction of *ts* (in the case of *tmp^{ts}* at permissive temperature) to be already sufficient for the loss of functional mitochondria. 2. Ascospores with the *tmp1* allele exhibit a higher rate of lethality than ascospores harboring the wild type allele. This may be explained as follows: Hybrids *tmp1/TMP1* were also heterozygous for the 5′-dTMP uptaking principle of strain 211-1aM (Brendel and Haynes [11]) and that coded by *TYP1* (Fäth *et al.* [1]). Ascospores may therefore have cell walls and membranes not permitting 5′-dTMP uptake though the ascospores may bear the genetic information for the 5′-dTMP uptaking principle(s). These uptaking principles might not phenotypically be established rapidly enough during germination and clonal growth of the ascospore. Hence the presence of a *tmp1* allele might lead to "thymineless death" as witnessed in mutant *typ1 tmp1-1* when deprived of 5′-dTMP (Brendel and Langjahr [12]).

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