SHORT COMMUNICATION

Isolation and Genetical Classification of Aromatic Amino Acid Auxotrophic Mutants in *Schizosaccharomyces pombe*

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Mutants of *Schizosaccharomyces pombe* with single and multiple requirements for aromatic amino acids were isolated and allocated to 15 loci. Difficulties in cultivation of many of these auxotrophs were largely overcome by using a minimal medium with L-glutamic acid as sole nitrogen source. The locus-typical growth factor requirements of mutants allowed a preliminary allocation of genes to reaction sequences in aromatic amino acid biosynthesis. Several explanations for the occurrence of mutants with locus-atypical growth factor requirements are discussed.

INTRODUCTION

We have attempted to isolate mutants of *Schizosaccharomyces pombe* auxotrophic for aromatic amino acids and aromatic vitamins. Mutants blocked in enzymes of the tryptophan-synthesizing branch of the pathway (Schweingruber & Dietrich, 1973) and others defective in isoenzymes controlling the first step of aromatic amino acid biosynthesis (Schweingruber & Wyssling, 1974) have already been isolated and characterized biochemically, but no mutants with multiple requirements for aromatic amino acids, as have been described in *Neurospora crassa* (Giles *et al.*, 1967), *Saccharomyces cerevisiae* (De Leeuw, 1968) and *Aspergillus nidulans* (Roberts, 1969), have been isolated. Here we report on the isolation and genetical classification of such *S. pombe* mutants as well as of others with single requirements for phenylalanine, tyrosine, tryptophan or *p*-aminobenzoic acid.

METHODS

Strains. The following strains of *Schizosaccharomyces pombe* stem from the silica gel stock collection of Professor U. Leupold, Institute of General Microbiology, University of Bern, Switzerland: wild-type strains 972 h⁻ and 975 h⁺; tryptophan auxotrophic strains, originally isolated by Dietrich (1970), *trp1A-41* h⁻/h⁺, *trp1B-4* h⁻/h⁺, *trp1C-3* h⁻/h⁺, *trp2-2* h⁻/h⁺, *trp3-23* h⁻/h⁺ and *trp4-47* h⁻/h⁺.

Media. Liquid yeast extract medium (YEL), malt extract agar (MEA) and minimal agar (MMA) were as described by Gutz *et al.* (1974). A slightly modified minimal medium (MMAg) was found to be more suitable for cultivation of aromatic amino acid auxotrophic mutants: in this, L-glutamic acid (10 g l⁻¹) replaced ammonium sulphate, Difco agar was used and the pH was adjusted to 5.5. Supplemented media (designated '+aro') contained L-phenylalanine, L-tyrosine and L-tryptophan (all at 10⁻³ M) and *p*-aminobenzoic acid (10⁻⁵ M), added to the medium after autoclaving. Plates with supplemented media were stored at 4 °C and were used within 2 weeks.

Mutant selection. Wild-type cells of *S. pombe* (972 h⁻ or 975 h⁺) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) to about 25 % survival: washed, exponentially growing cells from YEL (with 10 g glucose l⁻¹) were transferred to a freshly made solution of MNNG (2 mg l⁻¹ in 0.2 M-acetate buffer,

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Table 1. *Genetic classification and growth factor requirements of aromatic amino acid auxotrophs of S. pombe*

Different phenotypic classes of mutants from the same locus are listed in decreasing order of the frequency of mutants obtained.

Locus	Standard allele	Classes of growth factor requirements	Total no. of mutants
<i>aro3</i>	63	Phe, Tyr, Trp, Pab Phe, Tyr* Tyr**	117
<i>aro4</i>	98	Phe, Tyr, Trp, Pab Tyr* Phe, Tyr Phe or Tyr	75
<i>aro5</i>	110	Phe, Tyr Tyr**	17
<i>aro6</i>	789	Phe or Tyr**	2
<i>aro7</i>	353	Phe or Tyr** Tyr**	3
<i>aro8</i>	163	Tyr** (Phe-sensitive)	1
<i>tyr1</i>	62	Tyr	50
<i>tyr2</i>	78	Tyr** Phe, Tyr*	11
<i>phe1</i>	115	Phe** Phe or Tyr**	39
<i>phe2</i>	87	Phe**	30
<i>pab1</i>	47	Pab**	2
<i>trp1</i>	41, 4, 3	Trp	84
<i>trp2</i>	2	Trp	89
<i>trp3</i>	23	Trp Trp or Tyr**	52
<i>trp4</i>	47	Trp	33
Total			605

Phe, Phenylalanine; Tyr, tyrosine; Trp, tryptophan; Pab, *p*-aminobenzoic acid.

* Mainly leaky mutants. ** All leaky mutants.

pH 5.0) at a density of about 1.5×10^8 cells ml⁻¹ and incubated for 35 min at 30 °C. Treated cells were washed twice in similar acetate buffer, diluted in 0.14 M-KCl and immediately plated on MEA+aro (in early experiments) or on MMAG+aro (in later experiments). After 6 d (MEA+aro) or 10 d (MMAG+aro) at 30 °C, colonies were replicated to plates containing MMA+aro and MMA or MMAG+aro and MMAG, respectively. After 3 d at 30 °C, daughter plates were screened for clones requiring aromatic supplements. Mutant strains were kept on MEA+aro slants or, for long-term storage, on silica gel as described by Gutz *et al.* (1974). Strains were re-isolated every 6 months and tested for auxotrophy, haploidy, heterothallism and colony morphology. Some of the mutants were back-crossed to the wild-type for genotype purification and introduction of the opposite mating-type.

Growth factor requirements. Mutant cell material was inoculated on to MEA+aro plates in a pattern of 24 streaks per plate. After 3 d at 30 °C, the cultures were replicated on to the following media: MMA+tyrosine, MMA+phenylalanine, MMA+tryptophan, MMA+tyrosine+phenylalanine, MMA+*p*-aminobenzoic acid, MMA, MMA+aro. Growth response was scored qualitatively after 1, 2 and 4 d at 30 °C.

Genetic classification. Mutants were allocated to different loci using the criss-cross technique (Leupold, 1955). For this purpose, mutants were crossed first to each other and later to the standard alleles listed in Table 1.

RESULTS

A total of 799 mutant clones (colonies or colony sectors) with requirements for aromatic supplements were isolated by testing 163 500 colonies from five mutagenization experiments. Specific growth factor requirements and genetic classification were investigated for 605

of these strains (Table 1). The mutants were allocated unambiguously to 15 loci, of which 11 were newly discovered. All the tryptophan auxotrophs belonged to the four classes of *trp* loci reported by Schweingruber & Dietrich (1973). Experiments using MMAg+aro medium gave more *aro3*, *aro4* and *trp2* mutants but less *phe1*, *phe2* and *tyr2* mutants than those using MEA+aro medium. This was probably because single cells of some tightly blocked polyaromatic auxotrophs often failed to initiate growth on MEA+aro and were thus lost in the selection procedure.

Selected nonsense *aro3* mutants (Strauss, 1976), having strict aromatic growth factor requirements, needed *p*-aminobenzoic acid in addition to the three aromatic amino acids for optimal growth; addition of three other aromatic vitamins – *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde – had no effect. In preliminary experiments, high concentrations of ammonium ions and some amino acids (valine, leucine, histidine, phenylalanine, methionine) inhibited growth of these mutants. These effects were presumably due to inhibition of the uptake system for aromatic amino acids, as observed for polyaromatic auxotrophs in *Saccharomyces cerevisiae* (Greasham & Moat, 1973). Attempts to replace the aromatic amino acids by shikimic acid (300 mg l⁻¹) for supplementation of various *aro3* mutants failed.

Cross-feeding on minimal medium was observed only for some *trp* mutants (Strauss, 1976).

DISCUSSION

We isolated mutants of *S. pombe* with several types of growth factor requirements for phenylalanine, tyrosine, tryptophan and *p*-aminobenzoic acid, mapping at 15 different loci. While chromosome mapping has already been performed with most of these loci (Kohli *et al.*, 1977; A. Strauss, unpublished results), nothing is known with certainty on the gene–enzyme relationships of the 11 new loci. Nevertheless, the growth factor requirements suggest that all the isolated mutants are directly or indirectly blocked in some step of aromatic amino acid biosynthesis, provided this pathway proceeds through the same reactions in *S. pombe* as in other micro-organisms (Gibson & Pittard, 1968). Loci predominantly harbouring mutants with multiple requirements, such as *aro3*, *aro4* and *aro5*, are probably involved in steps prior to the branching-point intermediates chorismic acid (*aro3*, *aro4*) and prephenic acid (*aro5*), while loci with a majority of mutants needing only one growth factor, such as *phe1*, *phe2*, *tyr1*, *tyr2* and *pab1*, are presumably involved in steps leading from the branching-point intermediates to the corresponding end-products.

A detailed analysis of the genetic fine structure of the *aro3* locus has been presented elsewhere (Strauss, 1976). It is likely that this complex locus corresponds to the *arom* gene cluster of *Neurospora crassa* (Giles *et al.*, 1967) which codes for an enzyme complex bearing enzyme activity for steps two to six in early aromatic amino acid biosynthesis.

Some mutant phenotypes do not fit neatly into the above crude classification of loci for gene–enzyme relationships. At several loci, different classes of mutants with divergent growth factor requirements exist. A similar heterogeneity of mutant phenotypes had been observed for polyaromatic auxotrophic mutants of *Saccharomyces cerevisiae* (De Leeuw, 1968) and *Aspergillus nidulans* (Roberts, 1969), where it was suggested (De Leeuw, 1968) that all enzymes involved in aromatic amino acid biosynthesis form a loosely bound aggregate. Thus, mutants with atypical growth factor requirements might well be the result of some defect in aggregation of these enzymes. Another explanation for this phenomenon comes from the observation that most *aro3*, *aro4* and *aro5* mutants of *S. pombe* with aberrant phenotypes are incompletely blocked on minimal medium (Table 1). It is likely that these incompletely blocked mutants have reduced activity in one or several enzymes of the early biosynthesis. If these mutants produce the minimum required amount of only some of the end-products, the other end-products have to be supplied for full growth on minimal medium. This could be due either to unequal synthesis or to unequal

consumption of the end-products. The occurrence of tyrosine auxotrophic *aro3*, *aro4* and *aro5* mutants and phenylalanine and tyrosine auxotrophic *aro3* and *aro4* mutants can be explained in this way. The amount of synthesized tyrosine, and to a lesser extent phenylalanine, seems to be limiting in these mutants.

All *phe1* and *phe2* mutants of *S. pombe* possess a leaky phenotype (Table 1). Phenylalanine could possibly be synthesized in this organism by an alternative pathway. This would also account for the existence of mutants requiring phenylalanine or tyrosine which were found at several loci (Table 1). For *Geotrichum candidum*, an enzymic interconversion of phenylalanine to tyrosine has been reported (McEvoy, 1974). Alternatively, this leakiness could result from non-enzymic conversion of accumulated prephenic acid to phenylpyruvate (Friedrich & Schlegel, 1975).

Mutants in the *aro6*, *aro7*, *aro8* and *pab1* loci were isolated very rarely in this study, suggesting that they are mutated in a gene involved in regulation and/or that the isolation procedure was counterselective. The second explanation applies at least to the *aro8* mutant (which is phenylalanine-sensitive) and to the *pab1* mutants (which are probably fed by surrounding wild-type cell material).

The mutant isolation described here provides a starting point for studies of gene-enzyme relationships in aromatic amino acid biosynthesis of *S. pombe*.

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