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A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*

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The specific activity of branched-chain amino acid aminotransferase was highest when *S. cerevisiae* was grown in minimal medium containing a branched-chain amino acid as nitrogen source. Growth in complex media with glycerol or ethanol gave moderately high levels, whereas with glucose and fructose the specific activity was very low. Mutagenesis defined three genes (*BAA1* to *BAA3*) required for branched-chain amino acid catabolism. The *baa1* mutation reduced the specific activity of the aminotransferase, the stationary phase density in YEPD and caused gross morphological disturbance. Branched-chain amino acid aminotransferase is essential for sporulation.

Catabolism; Branched-chain amino acid; Aminotransferase; Mutation; *Saccharomyces cerevisiae*

1. INTRODUCTION

In the yeast, *Saccharomyces cerevisiae*, the catabolism of the branched-chain amino acids, leucine, isoleucine and valine, has been studied very little. It is not widely appreciated that *S. cerevisiae*, like other yeasts, can utilize any one of these three compounds as sole source of nitrogen [1–3]. The catabolism of amino acids has generally been regarded as a starvation response [4] and the relevant metabolic pathways as salvage pathways [5]. The well-characterized general amino acid permease (*GAP1* gene product), is a general uptake system. Specific uptake of branched-chain amino acids is catalysed by the high-affinity branched-chain amino acid permease which is encoded by the *BAP1* gene [6,7]. Until recently, the conventional wisdom was that catabolism then proceeds via a transaminase to yield the corresponding 2-oxoacid, decarboxylation by a 'carboxylase' to an aldehyde that is then reduced in a NADH-linked reaction producing the appropriate fusel alcohol [5,8]. The pathway has been called the 'Ehrlich Pathway' because the original concept was formulated by Ehrlich in 1904 [9] and slightly modified a few years later [10]. This pathway has never been proven to exist and the (mis-named) carboxylase has never been isolated. On the contrary, we have demonstrated recently that in *S. cerevisiae* decarboxylation of the 2-oxoacids derived from each of the branched-chain amino acids is achieved by a 2-oxoacid dehydrogenase which is a multienzyme complex very like that in mammals [11].

The metabolism of branched-chain amino acids is involved in the biosynthesis of fusel oils which are important constituents of all yeast-fermented alcoholic beverages and food products [12]. Because of the neglect of this aspect of metabolism in an organism as important as *S. cerevisiae*, and our conviction that the Ehrlich Pathway is incorrect, we have embarked upon a fresh survey of branched-chain amino acid catabolism in yeast. This paper outlines the isolation and characterization of some mutants which are defective in branched-chain amino acid catabolism. One mutation which affects branched-chain amino acid aminotransferase (EC 2.6.1.6) is described in detail.

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Abbreviations: EMS, ethylmethane sulphonate; Branched-chain amino acid aminotransferase – L-branched-chain amino acid, 2-oxoglutarate aminotransferase (EC 2.6.1.6).

2. MATERIALS AND METHODS

The transformable haploid IWD3 (*MATa ade1-100 his4-519 leu2-3,2-112 ura3-52*) and prototrophic haploid IWD72 (*MATα*) obtained originally from G. Reid (University of Edinburgh, UK) were used for most purposes. Strain 314 (*MATα ade5*) came from the authors' collection. Mutant C2901 (*MATα SUC2 mal gal2 CUP1 bap1*), which is defective in branched-chain amino acid permease, [7] was supplied by M.C. Kielland-Brandt (Carlsberg Laboratory, Copenhagen-Valby, Denmark). Homothallic diploid S41 (*MATa/MATα HO/HO arg4-1/arg4-1 cyh1/cyh1*) originated from H.O. Halvorson's laboratory (Brandeis University, Waltham, MA, USA).

The media and cultural conditions have all been described [11]. Sporulation was induced by the method of Fast [13]. Standard genetic techniques were used for mating, sporulation and dissection [14,15]. Crosses involving the homothallic diploid S41 were performed using cultures which had been sporulated prior to mating.

For enzyme assays cells were harvested by centrifugation, resuspended in buffer B (50 mM potassium phosphate, pH 7.4, 2 mM EDTA, 2 mM 2-mercaptoethanol) and disrupted using glass beads as described previously [16]. Aliquots of the homogenate produced in this way were used immediately as the source of enzyme. Branched-chain amino acid aminotransferase was assayed at 30°C using leucine (6.67 mM final concentration) as amino acid substrate in 200 mM-Tris/HCl buffer, pH 8.5, containing 6 mM 2-mercaptoethanol as described by Jenkins and Taylor [17]. 2-Oxoacid dehydrogenase was assayed as described previously [11].

For mutagenesis and the isolation of mutants, cells of strain IWD3 were grown in YEPD to $OD_{600\ nm}$ of 10 and treated with ethylmethane sulphonate as described by Henry and Keith [18]. Treated cells were diluted in YEPD to an $OD_{600\ nm}$ of 0.12 and incubated until the $OD_{600\ nm}$ became 1.17. The cells were then plated onto YEPD. The initial screening was for mutants which could grow on glucose minimal medium but not on 'BC minimal' medium [11] (which contains 2% valine as sole source of nitrogen). Strains showing the desired phenotype were retained, re-tested, and subsequently assayed for branched-chain amino acid aminotransferase and 2-oxoacid dehydrogenase activities.

3. RESULTS AND DISCUSSION

3.1. *The utilization by a wild-type strain of branched-chain amino acids as sole source of nitrogen*

The prototrophic wild-type strain, IWD72, was used for these preliminary studies to avoid possible complications due to the addition of amino acid auxotrophic requirements needed for growth in a minimal medium. We confirmed that any one of the three branched-chain amino acids will serve as sole source of nitrogen when glucose is the carbon source. The mean doubling time and final cell density were similar whichever branched-chain amino acid was being utilized in BC minimal media, or whether 2% (w/v) valine was used as a supplemented to conventional glucose minimal medium. A branched-chain amino acid did not serve as sole source of nitrogen and carbon. Small inocula (less than 1%, v/v) were used in all experiments to reduce 'carry over' of nitrogen or carbon substrates from the YEPD starter cultures.

3.2. *The effect of medium composition on the specific activity of branched-chain amino acid aminotransferase*

In stationary phase cells the specific activity of branched-chain amino acid aminotransferase was highest in cells which were grown in BC minimal medium containing 1% glucose or 2% fructose (Table I). Cells grown in BC minimal medium with either 2% glucose or 1% fructose, or in complex medium containing glycerol or ethanol, had moderately high levels. The lowest specific activity was found in cells grown in YEPD and YEPF. It is reasonable to expect significant levels of branched-chain amino acid aminotransferase in cells which have been grown in the presence of a branched-chain amino acid (BC minimal media): the presumed induction and/or activation of the enzyme thereby enabling the cells to utilize the substrate, however, other aspects of the regulation of this enzyme activity are not straightforward. For example, one might conclude from cells grown in minimal media that glucose causes some repression that is not caused by fructose; but this seems to be contradicted by a comparison of the specific activity in YEPF compared with YEPD.

3.3. *Isolation of mutants unable to utilize branched-chain amino acids*

3,700 colonies derived by plating onto YEPD the culture mutagenized with EMS were screened for their ability to grow on glucose minimal medium but not on BC minimal medium. From these, fourteen mutants were obtained with the desired phenotype after a second screening. In addition there were four other mutants which grew better on BC minimal medium than on glucose minimal medium. The members of this latter group were not studied further.

Mutant strains were crossed to the wild-type haploid 314 (*MATα ade5*) and dissected. The results were indicative that the phenotype was due to single gene mutations which were affecting nuclear-encoded genes, and that the mutations were recessive. Complementation tests were done on all fourteen mutations, and from the data obtained there were three complementation groups. These were named *baa1* to *baa3* to denote defects in branched-chain amino acid catabolism. Mutations affecting *BAA2* and *BAA3* were represented by only single alleles. Branched-chain amino acid aminotransferase and 2-oxoacid dehydrogenase were assayed in extracts from the 14 mutants and the wild-type parent strain grown for 48 h in YEPD. All of the strains identified as carrying *baa1* mutations had reduced levels of branched-chain amino acid aminotransferase activity. Mutant 1744 had the least with only 17% of the specific activity that was present in the wild-type parent grown under identical conditions. None of the mutants was defective in 2-oxoacid dehydrogenase. The biochemical nature of the defects in *baa2* and *baa3* mutants

Table I

Effect of medium composition on the specific activity of branched-chain amino acid aminotransferase

Growth medium	Specific activity (nmol of leucine consumed · min^{-1} · mg $protein^{-1}$)
YEPD	11 ± 4
YEPE	25 ± 5
YEPF	2 ± 1
YEPG	28 ± 1
BC minimal (1% glucose)	43 ± 1
BC minimal (2% glucose)	27 ± 2
BC minimal (1% fructose)	29 ± 1
BC minimal (2% fructose)	40 ± 2

Strain IWD72 was cultured to stationary phase in the different liquid media. The data are the means of four separate determinations (done on different days using different batches of cells) ± S.D.

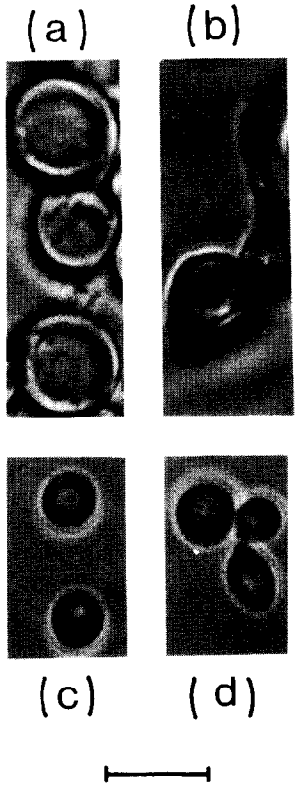

Fig. 1. The morphology of stationary phase wild-type and mutant haploids. A haploid bearing a *baa1* mutation (a,b) and wild-type strain IWD3 (c,d) were cultured to stationary phase in YEPD and then photographed. Bar = 10 μm.

remains unknown. The *bap1* mutant, C2901 (defective in the specific permease for branched-chain amino acids), grew well on BC minimal medium, so it seems likely that *baa2* and *baa3* do not affect the permease, rather components of the catabolic pathway that are further 'downstream' than 2-oxoacid dehydrogenase.

3.4. *Genetic characterization of the* baa1 *mutation which causes a loss of branched-chain amino acid aminotransferase activity*

Strain 1744 had the most stringent phenotype of all of the mutants isolated, so this was used as the starting point for more detailed genetic characterization of the *baa1* mutation. Three backcrosses were done to appropriate strains to ensure that the phenotype ascribed to the *baa1* mutation was indeed due to a single mutation and not caused by mutation at multiple loci. In twenty complete tetrads in which all four haploid spore progeny were viable the *baa1* mutation segregated 2:2, and inability to grow on BC minimal medium co-segregated with reduced specific activity of branched-chain amino acid aminotransferase. The mutation is probably a point mutation because spontaneous reversion occurs on growth in YEPD, resulting in a gradual increase in the specific activity of branched-chain aminotransferase in cultures with concomitant increased growth on BC minimal medium. The process was reversion and not extragenic suppression because crosses between cells which had regained the ability to grow on BC minimal and other wild-type strains failed to recover the *baa1* phenotype.

3.5. *Physiological characterization of the* baa1 *mutation*

In YEPD liquid medium *baa1* mutant strains only reached 70% of the stationary phase cell density attained by the wild-type. Microscopic examination after 48 h in YEPD medium, when both mutant and wild-type strains have ceased proliferating, revealed that the mutant appeared very different from the wild-type. Stationary phase cells from a *baa1*-bearing strain were all swollen (Fig. 1a). Roughly 3% were other bizarre shapes, typically approximately triangular or pyriform or spoon-shaped (Fig. 1b).

Diploids homozygous for the *baa1* mutation were asporogenous on potassium acetate sporulation medium, whereas heterozygous diploids could sporulate. This was an unexpected finding because it was not immediately obvious why this enzyme activity would be required under these conditions. Presumably one or more of the branched-chain amino acids is (are) deaminated prior to further degradation of the carbon skeletons or as part of a mechanism for the salvage of nitrogen in what is a nitrogen-depleted environment.

The asporogeny of *baa1/baa1* homozygous diploids and the reversion already noted presented a way of checking that the effect on sporulation was due to the

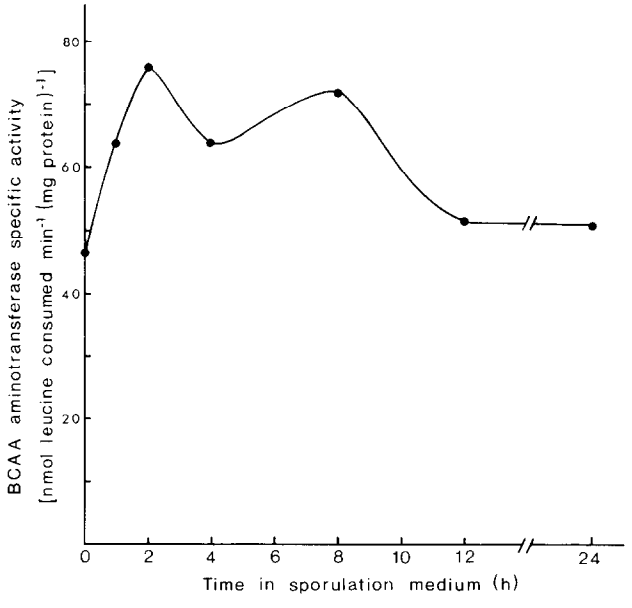

Fig. 2. The specific activity of branched-chain amino acid aminotransferase throughout sporulation. Diploid strain S41 was cultured in presporulation medium, harvested by centrifugation and then transferred to sporulation medium. The data are from a typical experiment and represent the means of duplicate determinations. The average variation between duplicates was 5%. At 24 h 76% of the cells were asci.

baa1 mutation and not another separate mutation in a sporulation-specific gene. Asporogenous diploid OD1 (*baa1/baa1*) was repeatedly sub-cultured to stationary phase three times in liquid YEPD and then aliquots were plated onto YEPD. These clones were replica-plated to sporulation medium and then stained with iodine vapour after 1 week. Colonies comprising sporulated cells stain differently from those which have not sporulated [19]. In six cases out of six the clones identified as sporulation-competent had regained the ability to grow on BC minimal medium and possessed higher levels of branched-chain aminotransferase activity than the original OD1 strain.

The inference that branched-chain amino acid aminotransferase may be required during sporulation prompted an examination of its activity throughout the course of sporulation in wild-type diploid strain S41. The specific activity of this enzyme increased by as much as 70% in the first 2 h after transfer to sporulation medium (Fig. 2). Thereafter, the specific activity declined via a second maximum around 8 h, such that by 24 h the specific activity was roughly similar to that when the cells were first transferred to sporulation medium. The increase in specific activity during the first 2 h was completely abolished by the presence of cycloheximide (100 $\mu g \cdot ml^{-1}$) (data not shown). Either the increase in activity during the initial stages of sporulation requires the synthesis of new aminotransferase molecules, or an inhibitor of the aminotransferase could be inactivated by another newly synthesized enzyme; however, the specific activity of branched-chain amino acid aminotransferase increased in a similar manner in haploid strain IWD72. Hence, the increase in branched-chain amino acid aminotransferase is not a sporulation-specific event but the enzyme is essential for sporulation because *baa1* homozygous diploids cannot sporulate.

One of the earliest metabolic changes that occurs upon initation of sporulation is the synthesis of a large amount of glutamate [20,21]. The carbon atoms derive from the acetate of the sporulation medium, but the origin of the amino groups has always been unclear. The timing of the increase in branched-chain amino acid aminotransferase matches that of the bulk of new glutamate synthesis and is mirrored by the intracellular levels of branched-chain amino acids ([20,22] and unpublished observations), and could certainly provide some, if not all, of the amino groups required for the synthesis of glutamate.

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