JOURNAL OF BACTERIOLOGY, Mar. 1984, p. 958–961
0021-9193/84/030958-04$02.00/0

Vol. 157, No. 3

Asparaginase II of *Saccharomyces cerevisiae*: Positive Selection of Two Mutations That Prevent Enzyme Synthesis

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Received 29 August 1983/Accepted 8 December 1983

A positive selection method, D-aspartic acid β-hydroxamate resistance, was used to isolate *Saccharomyces cerevisiae* strains lacking the ability to synthesize asparaginase II. Of 100 such mutant strains, 93 exhibited mutations which were allelic with *asp3*, a previously characterized mutation. The other seven strains carried a new mutation, *asp6*. The *asp6* mutation segregated 2:2 in *asp6* × wild-type crosses and assorted from the *asp3* mutation in *asp6* × *asp3* crosses. All seven *asp6* mutant isolates reverted at a relatively high frequency, whereas the *asp3* mutant isolates did not revert under the same conditions. Various independent *asp3* isolates were mated to give heteroallelic diploids, which when sporulated and spread on D-asparagine medium yielded no recombinant strains.

Asparaginase II is a yeast exoenzyme which is secreted into the cell wall in response to nitrogen limitation (5–9, 11, 12, 15–17, 20–22, 25). The genetics of *Saccharomyces cerevisiae* asparaginase II have been previously characterized with wild-type *S. cerevisiae* strains which either fail to synthesize the enzyme or exhibit a reduced synthetic capacity (5, 11, 12, 20). Yeasts carrying the *asp2* mutation can synthesize only 20 to 30% of the normal enzyme levels. Strains with the *asp3* mutation do not synthesize the enzyme. Yeasts with the *asp4* mutation have a greatly reduced ability to synthesize the enzyme when growing exponentially on ammonium ion but exhibit normal enzyme levels when starved for nitrogen.

The mutations described above were isolated from the genetic background of various wild-type *S. cerevisiae* strains with no mutagenesis being employed. We wanted to determine whether other asparaginase mutations could be obtained through the use of chemical mutagenesis. In this study, we used ethanemethylsulfonate (EMS) mutagenesis and a positive selection method to isolate *S. cerevisiae* strains with a reduced capacity to synthesize asparaginase II.

The strain designations and source or method of derivation are given in Table 1.

The standard chemically defined medium contained, per liter: 30 g of D-glucose, 2 g of yeast nitrogen base (without amino acids and ammonium sulfate), 1.32 g of ammonium sulfate and 1.69 g of L-glutamic acid (monosodium salt). The cells were harvested by centrifugation after growth at 23°C for 12 to 16 h when the absorbance at 660 nm was 0.1 to 0.2. The harvested cells were used immediately.

The derepression medium contained 3% D-glucose and 20 mM potassium phosphate buffer, pH 7.0. Cells were suspended in derepression medium to an absorbance of 0.4 at 660 nm (ca. 0.12 mg/ml [dry weight]) and allowed to incubate at 30°C for 4 h.

Whole-cell suspensions were assayed at 30°C for the ability to convert L-asparagine and hydroxylamine into L-aspartic acid β-hydroxamate as described previously (6). Whole-cell asparaginase II activity is reported as nanomoles of the β-hydroxamate synthesized per minute per milligram of cells (dry weight).

Genetic analysis was performed by the methods of Mortimer and Hawthorne (18). Diploids were obtained by mixing strains of opposite mating types on yeast extract-peptone-dextrose plates. After 4 h of incubation at 27°C zygotes were isolated by micromanipulation. Sporulation of diploid strains and tetrad analysis were conducted by using published methods (18).

For the selection of spontaneous revertants of asparaginase II mutants, yeast strains carrying *asp3* or *asp6* mutations were grown to the late exponential phase on the standard minimal medium and then plated on minimal medium containing, per liter: 30 g of D-glucose, 2 g of yeast nitrogen base (without amino acids and ammonium sulfate), and 5 mM D-asparagine to 5×10^7 cells per plate for *asp3* mutants or 1×10^6 cells per plate for *asp6* mutants. The plates were incubated at 27°C for 7 days and scored for the appearance of visible colonies.

Selection of asparaginase II mutants. *S. cerevisiae* strains exhibiting greatly reduced levels of asparaginase II activity were isolated from parental strain DJ2-23c (*MATα gap1 asp1*). The parental strain can synthesize high levels of asparaginase II but lacks the ability to synthesize asparaginase I, the internal form of yeast asparaginase (5, 11, 14). In addition, it cannot transport D-amino acids because of the *gap1* mutation which inactivates the general amino acid transport system (26).

Strain DJ2-23c was mutagenized with EMS and spread to ca. 5×10^6 cells per plate on minimal glucose medium containing, per liter: 30 g of D-glucose, 2 g of yeast nitrogen base (without amino acids and ammonium sulfate), 5 mM L-proline and 0.5 mM D-aspartic acid β-hydroxamate. The D-aspartic acid β-hydroxamate can be cleaved by asparaginase II to D-aspartic acid and hydroxylamine. Because of the *gap1* mutation, neither D-asparagine nor D-aspartic acid can enter yeast cells (26). However, hydroxylamine can enter the cells via the methylamine-ammonia transport system and is highly toxic (24). Consequently, wild-type strains which synthesize asparaginase II produce hydroxylamine and grow poorly on this medium, but cells with reduced levels of asparaginase II activity can grow well and after 6 to 10 days form large colonies. These large colonies were selected, streaked, and grown to single isolated colonies on a second β-hydroxamate plate before biochemical analysis.

Approximately 1,000 yeast strains were selected which grew rapidly on minimal agar plates containing D-aspartic acid β-hydroxamate. About 30% of the total colonies select-

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TABLE 1. Asparaginase II mutants fall into two classes, *asp3* and *asp6*

Haploids	Relevant Genotype	Source	Haploid activity levels	Diploid activity levels[a] (× X2180-1A) (wt)	(× XE223-1D) (*asp3*)	(× XE221-2B) (*asp2*)	(× A-AHRIV-46)[b] (*asp6*)
X2180-1A	(*MAT***a**)	Berkeley collection	71	—	—	—	—
X2180-1B	(*MAT*α)	Berkeley collection	62	74	35	43	48
DJ2-23c	(*MAT*α)	Our laboratory	65	71	33	43	48
XE221-2A	(*MAT*α *asp2*)	Gary Jones; from wild-type strains lacking asparaginase II activity[c]	9	43	31	11	34
XE221-2B	(*MAT***a** *asp2*)		11	—	—	—	—
XE223-1C	(*MAT*α *asp3*)		<2	42	<2	31	33
XE223-1D	(*MAT***a** *asp3*)		<2	—	—	—	—
AHRIV-46	(*MAT*α *asp6*)	Our laboratory; by EMS mutagenesis of DJ2-23C and β-hydroxamate selection	<2	52	32	35	<2
AHRIV-1	(*MAT*α *asp3*)	Our laboratory; by EMS mutagenesis of DJ2-23C and β-hydroxamate selection	<2	56	<2	33	—

[a] Crosses designated—either do not mate or were not tested.
[b] Strain A-AHRIV-46 was derived by crossing AHRIV-46 with X2180-1A and selecting as asparaginase II-negative, *MAT***a** segregant.
[c] G. E. Jones, Department of Biology, University of California, Riverside, Calif.

ed had low asparaginase II activity (<50% of wild type), whereas the other 70% of the colonies exhibited normal asparaginase II synthesis. These latter strains appeared to be hydroxylamine transport mutants, defective in the methylamine-ammonia system (24).

One hundred mutant strains which exhibited <5% wild-type asparaginase II activity and also gave normal growth rates on minimal glucose ammonia medium were used for further biochemical and genetic characterization.

Table 1 gives asparaginase II activity levels obtained with representative strains of the newly isolated mutants. The mutants were tested directly as haploids and also as diploids after mating with previously characterized tester strains. The new isolates fell in two mutational classes. The majority (93%) carried a mutation which is allelic with the *asp3* (5, 11, 12). The others (7%) carried a new mutation which was designated *asp6*.

Both *asp3* and *asp6* mutations were recessive in diploids formed by mating with a wild-type strain capable of asparaginase II synthesis. The level of activity in these heterozygous diploids was about 70% of the control activity found in a homozygous wild-type tester strain. This lowered activity may result from a gene dosage effect. The *asp3* isolates were identified by their failure to complement an *asp3* tester strain. The new *asp3* mutants exhibited complementation with an *asp2* strain (11, 12). The level of enzyme expression in *asp3*/*asp2* diploids was about 50% of that found with the diploid wild-type tester. This low activity may reflect both gene dosage and possibly a suppression of activity due to factors in the background of the *asp2* tester strain. (The *asp2* and *asp3* testers used in this study gave 60% of the control activity in heterozygous diploids formed with the standard wild-type tester strain.)

The *asp6* strains complemented both *asp3* and *asp2* testers. Diploids of *asp6* × *asp3* and *asp6* × *asp2* matings exhibited 50% of the control activity. These low activity levels probably reflected the same factors causing reduced activity in the *asp3*/*asp2* diploid.

Segregation analysis of *asp3* and *asp6* mutations. Segregation analysis was performed with the new *asp3* and *asp6* isolates. Typical results are shown in Table 2. Both *asp3* and *asp6* mutations exhibited 2:2 segregation in mutant/wild-type crosses, indicating that these mutations are the result of alteration in individual nuclear genes. When segregation analysis was performed on spores from a diploid heterozygous for both *ASP3* and *ASP6*, we found 4:0, 3:1, and 2:2 (mutant-to-wild-type) segregation patterns in various tetrads. This indicated that the two mutations assorted from each other and thus did not occur in the same or in tightly linked genes. However, the segregation pattern revealed a high frequency of 4:0 segregation, suggesting that the two mutations are weakly linked on the same chromosome.

In a separate study, the *asp3* mutation was analyzed for linkage with two centromere-linked mutations, *trp1* and *leu2*. The data indicated that the *ASP3* gene (and thus probably the *ASP6* gene) was not centromere linked (data not shown).

Attempted identification of structural gene mutations. We have made a number of attempts to identify structural gene mutations for asparaginase II. With most enzymes, this can be accomplished by characterizing a leaky mutation which alters the structure or function of the enzyme product. With such a mutation the enzyme may exhibit an altered K_m for one of its substrates or increased temperature lability. We

TABLE 2. Segregation pattern of *asp3* and *asp6* mutations in various crosses

Parental diploid strain	No. of tetrads	No. of tetrads exhibiting segregation pattern[a] 4:0	3:1	2:2
X2180-1A (wt) × AHRIV-46 (*asp6*)	20	0	1	19
X2180-1A (wt) × AHRIV-1 (*asp3*)	24	0	1	23
XE223-1C (*asp3*) × AHRIV-46 (*asp6*)	69	31 (14)[b]	24 (56)	14 (14)

[a] Mutant:wild type.
[b] Numbers in parentheses indicate the predicted frequency for two unlinked mutations.

have examined over 200 D-aspartic acid β-hydroxamate resistant *S. cerevisiae* mutants which are leaky (5 to 20% control level) for asparaginase II. Using these strains, we were unable to identify a K_m or temperature-sensitive mutation (data not shown).

In addition to direct selection of β-hydroxamate-resistant mutants, we used a second strategy to isolate leaky yeast strains. The 100 *asp3* and *asp6* strains were plated onto D-asparagine containing medium to select spontaneous revertant colonies which could grow with D-asparagine as a nitrogen source. Table 3 gives typical results from this selection procedure. The seven *asp6* isolates gave high rates of spontaneous reversion. Approximately 100 of these revertants exhibiting asparaginase II activity of 5 to 25% of the control were assayed for enzyme with altered K_m or temperature sensitivity. No changes in these parameters could be detected in any of the revertant strains (data not shown).

With the *asp3* isolates, a different pattern was evident. None of the 93 *asp3* strains tested gave any spontaneous revertants. Treatment with various chemical mutagens also failed to produce revertant strains. In one further attempt to reestablish asparaginase II activity in the *asp3* isolates, various independent isolates were mated and then sporulated to induce meiotic recombination (10). To accomplish this, 12 *asp3* isolates were first mated to a wild-type strain; the resulting diploids were sporulated, and the 12 *asp3* mutations were isolated in *MAT***a** haploid segregants. From these 12 strains, *asp3* × *asp3* heteroallelic crosses were made with the 12 original isolates in *MAT*α strains. The diploids were sporulated and the spores plated on minimal medium containing D-asparagine as the sole nitrogen source. Although this procedure has the potential for producing high frequencies of recombination, e.g., between 0 to 5,000 recombinants per 10^5 asci were reported for various alleles of the *ade8* gene (10), in our study, no recombinants could be detected after plating 5×10^6 asci from the various *asp3* × *asp3* crosses.

Function of the *ASP3* gene. In previous reports, we have suggested that *ASP3* might be the structural gene for asparaginase II; at that time, *ASP3* was the only known gene in which mutation could cause the complete loss of asparaginase II activity (5). In addition, *asp3* mutant alleles occurred in many wild-type strains, and it seemed plausible that these strains lacked the structural gene for asparaginase II. An alternate possibility, which we thought less likely, was that these strains had the asparaginase II structural gene but were silent in its expression because of a regulatory mutation.

At present, it is not clear whether either the *ASP3* or the newly defined *ASP6* gene correspond to the structural gene for asparaginase II. Despite rigorous attempts, we have been unable to identify any alleles of *ASP3* or *ASP6* which result in the production of a modified form of asparaginase II. Other genetic properties of the *asp3* mutations appeared to be unusual for structural gene mutations. For example, none of the 93 isolates carrying the *asp3* mutation could be induced to revert. Since EMS mutagenesis was used to produce these strains, we expected to isolate point mutations as the predominant products. If most of our 93 independent *asp3* isolates carried point mutations in a structural gene, at least a few revertable alleles would be expected. Furthermore, when heteroallelic *asp3* × *asp3* diploids were sporulated, we could not recover any recombinants which expressed asparaginase II activity. The absence of recombinants from independently isolated mutations suggested that we were not working with single point mutations located at random along a structural gene, but rather with a more unusual type of mutation which must occur repeatedly in the various mutant isolates of the *ASP3* locus. A deletion mutation covering an extensive portion of the genome could account for both the lack of reversion and the absence of meiotic heteroallelic recombination.

TABLE 3. Reversion of asparaginase II mutant strains

Strains	Revertant Colonies/ 5×10^7 Cell Plated[a]
XE223-1C (*MAT*α *asp3*)	0
XE223-1B (*MAT***a** *asp3*)	0
AHRIV-46 (*MAT*α *asp6*)	6.9×10^3
AHRIV-1 (*MAT*α *asp3*)	0

[a] Cells were plated on minimal D-asparagine medium and scored after a 7-day incubation (10^6 cells were plated for *asp6* strains; 5×10^7 cells were plated for *asp3* strains).

One possible explanation for our results may be that asparaginase II is encoded by more than one structural gene. By studying only *S. cerevisiae* strains that were at least 80% inactivated for the enzyme, we may have excluded structural gene point mutations which would cause a reduction in activity of 50% or less. This hypothesis is consistent with recent data from our laboratory indicating that purified fractions of asparaginase II contain more than one protein band (manuscript in preparation). It is also analogous with the results obtained with the yeast exoenzymes, invertase and acid phosphatase, which are known to be encoded by multiple structural genes and which exhibit protein heterogeneity in purified preparations (1–4, 23, 27). Finally, it is interesting that with maltase, which is the product of multiple structural genes, no structural gene mutants have ever been identified (19).

To fully understand the behavior of the *asp3* mutations, it will be necessary to compare the gene structure in the *ASP3* parent and in various *asp3* mutant *S. cerevisae* strains.

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