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L-Asparagine Auxotrophs of *Saccharomyces cerevisiae*: Genetic and Phenotypic Characterization

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L-Asparagine auxotrophy in *Saccharomyces cerevisiae* is the result of mutation in each of two unlinked cistrons, *ASN1* and *ASN2*. Mutation in only one of these cistrons yields growth indistinguishable from that of wild-type cells under a variety of nutritional stresses. Relatively high concentrations of L-asparagine are required to permit maximal growth of the auxotrophs, and the amino acid requirement cannot be satisfied by a variety of other amino acids that serve as nitrogen sources for cell growth. Although reversion of the mutations can occur, haploid populations of cells containing only low frequencies of prototrophs can be maintained easily. In diploid cells heteroallelic for certain combinations of alleles of the two genes, mitotic recombination gives rise to prototrophic cells that accumulate to high frequency in populations of the cells.

Mechanisms by which the synthesis, utilization, and degradation of L-asparagine are regulated are of key importance to the growth and development of cells of both animals and plants. In animal cells of several types, a variety of cellular processes involving the induction or continuation of mitosis are inhibited or delayed when those cells are treated with L-asparaginase (EC 3.5.1.1), which hydrolytically cleaves L-asparagine to yield L-aspartate and ammonium ion (reviewed in reference 14). In plants, especially in the legumes, L-asparagine synthesis, transport, and degradation play a central role in the fixation and assimilation of atmospheric nitrogen and in the development of bacteroid nodules and seeds (1, 13).

We have undertaken a biochemical genetic study of L-asparagine metabolism in the yeast *Saccharomyces cerevisiae*. This species provides an ideal model system for studying the relationship between asparagine and nitrogen metabolism in eucaryotes, because it possesses at least two enzymes (L-asparaginase I and asparaginase II) by which asparagine can be degraded and used as a nitrogen source, and it must possess pathway(s) by which this amino acid can be synthesized. The characteristics of the two asparaginases indicate that, in this species, the enzymes have evolved to satisfy two different requirements for cellular homeostasis. L-Asparaginase I, which is constitutive and is coded for by a single structural gene (7, 10, 11), appears to interact primarily with intracellular pools of L-asparagine, perhaps functioning as a part of a mechanism by which these pools are controlled. Asparaginase II, which is derepressible by nitrogen starvation and requires functional product of at least two genes (3, 4, 8, 9), apparently functions either as a sensor/effector related to the state of nitrogen metabolism in the cell or as an element that responds to a separate sensor of nitrogen balance in the cell. Almost certainly, these regulatory mechanisms will involve the cells' ability to synthesize L-asparagine and thereby convert nitrogen into the amide form. Because of this, it is essential that mutants be isolated that are unable to synthesize this amino acid so the effects of this function on overall nitrogen metabolism can be determined. Previous attempts to isolate such mutants of yeast without applying enrichment procedures have failed, all such putative mutants appearing to be highly unstable (10). In this paper, I report the successful selection of L-asparagine auxotrophs of *S. cerevisiae* and describe their genotypic and growth properties.

MATERIALS AND METHODS

Yeast strains. The yeast *Saccharomyces cerevisiae* was used. All strains except strain MC-6A were derived from stocks harbored in the Yeast Genetics Stock Center, University of California, Berkeley, Calif. Strain MC-6A (a *ino1-13 ino4-8*) was kindly provided by Susan A. Henry. Genotypes and phenotypes are given as strains are mentioned in the text. Gene symbols have been described (10).

Media. YEPD contained, in 1 liter of distilled water, yeast extract (Difco), 10 g; Bacto peptone (Difco), 20 g; and dextrose, 20 g. Minimal medium with vitamins (MV) contained, per liter of distilled water, yeast nitrogen base without amino acids (Difco), 6.7 g; and dextrose, 20 g. Minimal medium with vitamins lacking ammonium sulfate (MV-NH_3) contained, in 1 liter of

distilled water, yeast nitrogen base without amino acids and ammonium sulfate (Difco), 1.45 g; and dextrose, 20 g. Solid media contained 20 g of agar (Difco) per liter of distilled water.

Concentrated stocks of amino acid supplements were sterilized by filtration and added to other media when required. Liquid MV and MV-NH_3 were prepared from filter-sterilized, concentrated stock solutions of Bacto yeast nitrogen base without amino acids (Difco) or Bacto yeast nitrogen base without amino acids and ammonium sulfate (Difco), respectively. Stocks were added to autoclaved distilled water cooled to room temperature to prepare working solutions.

Growth of cells. Cells were grown at 29 ± 1°C. Cells in liquid media were grown on rotary shakers operated at 200 rpm. Culture turbidity at 520 nm was used to indicate cell density. Turbidity was measured in 22-mm (OD) culture tubes in a Spectronic-20 (Bausch and Lomb) spectrophotometer.

Genetic methods. Yeast crosses and segregational analyses were carried out as previously described (12). Asci were dissected directly onto enriched medium contained in plastic petri dishes (15 by 100 mm). Diploid strains always were initiated by using a micromanipulator to isolate single zygotes from mating mixtures. Diploidy was ascertained by sporulating putative diploid strains.

Mutant selection. A culture of cells of strain MC-6A (an inositol auxotroph) was initiated from a single colony. The cells then were treated with 3% ethyl methane sulfonate (10) to permit approximately 20% survival. This culture was split into two cultures immediately after mutagen treatment, and the two cultures then were subjected to inositol-less death counterselection essentially as described by Henry et al. (6). Cells from the mutagen-treated cultures were grown in MV medium (which contains inositol) supplemented with L-asparagine (2 mM) and then incubated for 6 h in MV medium supplemented with L-aspartate (0.75 mM). In the latter medium, any L-asparagine auxotrophs present should cease growth, but auxotrophs whose requirements can be met by either L-asparagine or L-aspartate (e.g., glutamateoxalacetate transaminase mutants) should continue to grow and be selected against during subsequent inositol starvation. Cells from these cultures were then incubated for 24 h in MV medium lacking inositol but supplemented with L-aspartate (0.75 mM). After 24 h, survival in these cultures was approximately 0.8%. Inositol and L-asparagine (2 mM) then were added to allow possible L-asparagine auxotrophs to grow. Cells from these final cultures were plated onto MV plates supplemented with L-asparagine (2 mM) and allowed to grow into colonies. Colonies were replica plated onto MV plates and onto MV plates supplemented with L-aspartate (0.75 mM). Cells that were able to grow when provided with L-asparagine but that could not grow on minimal medium or minimal medium supplemented with L-aspartate were picked and tested further.

RESULTS

Selection of L-asparagine-requiring mutants. From each of the two cultures subjected to enrichment, approximately 20,000 colonies were screened for L-asparagine auxotrophy. From culture no. 1, only a single putative mutant was found. From culture no. 2, two putative mutants were found. These strains were labeled Asn-1-97b, Asn-2-27, and Asn-2-39, respectively. After the mutants were isolated, they were repeatedly backcrossed to our standard laboratory wild-type strains, which themselves are the products of repeated backcrosses to strain S288C (α). During these crosses, segregants were chosen that did not require inositol for growth, and from these, auxotrophic segregants of each mating type were selected for further study.

Recessiveness of L-asparagine auxotrophy. Heterozygous or homoallelic diploid cells derived from crossing mutant haploids with wild-type cells or among themselves were tested for ability to grow without added L-asparagine. Homoallelic mutant diploids required L-asparagine, but cells heterozygous for the requirement did not. Therefore, asparagine auxotrophy is recessive to prototrophy.

Complementation analysis. Diploid cells heteroallelic or homoallelic for mutations engendering L-asparagine auxotrophy were tested for ability to grow on unsupplemented minimal medium. None of these diploids could grow without exogenous L-asparagine. Therefore, mutations causing auxotrophy in the three original mutants all arose in the same cistrons (see below).

Segregation of L-asparagine auxotrophy. Diploid cells heterozygous for L-asparagine auxotrophy were sporulated, asci were dissected, and haploid segregants from the crosses were tested for L-asparagine requirement. Several such crosses were performed with auxotrophs chosen from sequential backcrosses to wild type; ascus analyses and segregational data from these crosses are presented in Table 1. The pattern of segregation can best be interpreted by assuming that mutations in two genes are required to confer L-asparagine auxotrophy. Furthermore, a haploid cell must carry a mutant allele of both these genes for auxotrophy to be expressed. Therefore, parental-ditype (PD) asci from the crosses show 2+:2− segregation, nonparental-ditype (NPD) asci show 4+:0− segregation, and tetratype (T) asci show 3+:1− segregation, where "+" represents prototrophic and "−" represents auxotrophic growth. I have tentatively named these cistrons *ASN1* and *ASN2*. Alleles originally carried by Asn-1-97b are called *asn1-1 asn2-1*, those carried by Asn-2-27 are called *asn1-2 asn2-2*, and those carried by Asn-2-39 are called *asn1-3 asn2-3*.

Assignment and confirmation of genotypes. To confirm that mutations in two genes

TABLE 1. *Ascus analysis for segregation of* L-*asparagine auxotrophy*

Original parent[a]		Ascus type			Degrees of freedom	Chi-square[b]
		PD (2+:2−)	NPD (4+:0−)	T (3+:1−)		
Asn-1-97b	Observed	33	22	113		
	Expected (1:1)[c]	27.5	27.5		1	2.20
Asn-2-27	Observed	12	9	39		
	Expected (1:1)	10.5	10.5		1	0.428
Asn-2-39	Observed	7	7	22		
	Expected (1:1)	7	7		1	0.000
Pooled	Observed	52	38	174		
	Expected (1:1)	45	45		1	2.178

[a] Numbers represent the combined results of several backcrosses of auxotrophs to wild type.
[b] None of these chi-squares is significant at the 95% confidence level.
[c] Numbers represent those expected if two alleles of each of two unlinked genes were segregating in the crosses.

are involved in the expression of auxotrophy, an extensive analysis of crosses was performed based on the following rationale. From a T ascus derived from a cross of a presumptive double mutant (*asn1 asn2*) to wild type (*ASN1 ASN2*), haploid strains of four genotypes should be derived: *ASN1 ASN2, asn1 ASN2, ASN1 asn2,* and *asn1 asn2*. Only the last of these should exhibit auxotrophy. If a presumptive *asn1 ASN2* strain is crossed with a presumptive *ASN1 asn2* strain, PD, NPD, and T asci in the ratio 1:1:4 should result. When the auxotrophic strain (presumably *asn1 asn2*) is crossed with a known wild-type strain, 1PD:1NPD:4T ascus ratios should result. When the presumed *ASN1 ASN2* strain is crossed with the presumed *asn1 asn2* strain, 1PD:1NPD:4T ascus ratios again should be observed, and when a presumed single mutant (e.g., *ASN1 asn2*) is crossed with the auxotrophic strain, all asci should contain two prototrophic and two auxotrophic haploid segregants. Finally, when a presumed single mutant (e.g., *asn1 ASN2*) is crossed with the presumed *ASN1 ASN2* strain, all haploid segregants should be prototrophic. To test these predictions, all possible crosses were made using haploids derived from a T ascus from cross XE299 (Table 2). Of the four strains derived from this ascus, only XE299-1A was auxotrophic. Results of crosses XE370 through XE374 showed that XE299-1A is mutant in both genes, that XE299-1B carries a mutation in only one of the genes, that XE299-1C carries a mutation in the other of the two genes, and that XE299-1D is doubly wild type. The mutation in XE299-1B was defined to be *asn1-1*, and the mutation in XE299-1C was defined to be *asn2-1*. Thus, the genotypes of the four strains derived from this tetrad are as given in Table 2. Similar analyses were performed for genes derived from the original mutants Asn-2-27 and Asn-2-39 with similar results.

After XE299-1B and XE299-1C were defined to be carrying *asn1-1* and *asn2-1*, respectively, genotypes of singly mutant strains carrying other alleles were determined by crossing these strains with XE299-1B or XE299-1C or to singly mutant strains derived from them. For example, strains XE304-1A and XE304-1B were shown to be carrying single mutations in *ASN1* or *ASN2* by crossing them with an auxotroph (crosses XE317 and XE318 in Table 2). When XE304-1A was crossed with XE299-1B, 1PD:1NPD:4T ascus ratios were observed, and when it was crossed with XE315-1A (known to be of genotype *ASN1 asn2-1*), only prototrophic segregants were observed (crosses XE321 and XE377 in Table 2). Thus, XE304-1A is of gentoype *ASN1 asn2-3*. Similar crosses (XE322 and XE345) showed that XE304-1B is of genotype *asn1-3*. That in fact these strains each carry one mutation in a different gene of the pair was confirmed by crossing derivatives of them and showing that segregation of auxotrophy occurred in the expected 1PD:1NPD:4T ascus ratios (crosses XE375 and XE376 in Table 2). Similar analyses of crosses were used to determine and confirm genotypes of all strains used in this study.

Stability of mutations conferring L-asparagine auxotrophy. Because of previously noted instability of putative L-asparagine auxotrophs (10), stability of mutants isolated in this study was determined both in haploid and diploid auxotrophs. To determine the stability of mutations in *ASN1* and *ASN2* in haploid cells, strains carrying mutations in both genes were plated onto MV plates, and colonies visible after 3 and 6 days of incubation were counted. The results of one such experiment are presented in

TABLE 2. *Determination of genotypes of strains carrying mutant alleles of ASN1 and/or ASN2*

Cross no.	Parents	Segregation pattern					
		Predicted[a]			Observed[b]		
		4+:0−	3+:1−	2+:2−	4+:0−	3+:1−	2+:2−
XE370[c]	XE299-1B (**a** *asn1-1 ASN2*)/XE299-1C (α *ASN1 asn2-1*)	3.8	15.3	3.8	5	13	5
XE371	XE299-1A (**a** *asn1-1 asn2-1*)/S288C (α *ASN1 ASN2*)	3.7	14.7	3.7	1	16	5
XE372	XE299-1A (**a** *asn1-1 asn2-1*)/XE299-1D (α *ASN1 ASN2*)	2.8	11.3	2.8	1	14	2
XE373	XE299-1A (**a** *asn1-1 asn2-1*)/XE299-1C (α *ASN1 asn2-1*)	0	0	19	0	0	19
XE374	XE299-1B (**a** *asn1-1 ASN2*)/XE299-1D (α *ASN1 ASN2*)	23	0	0	23	0	0
XE317	XE304-1A (α *ASN1 asn2-3*)/XE304-2A (**a** *asn1-3 asn2-3*)	0	0	19	0	0	19
XE318	XE304-1B (α *asn1-3 ASN2*)/XE304-2A (**a** *asn1-3 asn2-3*)	0	0	23	0	0	23
XE321	XE304-1A (α *ASN1 asn2-3*)/XE299-1B (**a** *asn1-1 ASN2*)	3.3	13.3	3.3	3	11	6
XE377	XE304-1A (α *ASN1 asn2-3*)/XE315-1A (**a** *ASN1 asn2-1*)	21	0	0	21	0	0
XE322	XE304-1B (α *asn1-3 ASN2*)/XE299-1B (**a** *asn1-1 ASN2*)	13	0	0	13	0	0
XE345	XE304-1B (α *asn1-3 ASN2*)/XE315-1A (**a** *ASN1 asn2-1*)	3.8	15.3	3.8	4	11	8
XE375	XE304-1A (α *ASN1 asn2-3*)/XE318-1A (**a** *asn1-3 ASN2*)	3.3	13.3	3.3	3	15	2
XE376	XE304-1B (α *asn1-3 ASN2*)/XE317-1B (**a** *ASN1 asn2-3*)	3.5	14	3.5	3	15	3

[a] Predicted numbers of asci assuming that genotypes are as given and that *ASN1* and *ASN2* are unlinked.

[b] Only complete tetrads were included. In every cross, spore viability exceeded 90%. In no cross was the deviation from expected values significant at the 95% level of confidence.

[c] Except for S288C, all strains used in crosses XE370 to XE374 were from a single tetratype ascus from cross XE299, the parents of which were strains XE261-2C (**a** *asn1-1 asn2-1*) and S288C (α *ASN1 ASN2*). XE261-2C was an L-asparagine auxotroph derived from sequential backcrosses of Asn-1-97b to standard wild-type strains.

TABLE 3. *Frequencies of revertants in populations of* L-*asparagine auxotrophs*[a]

Strain	Genotype	Total no. of cells plated	Total no. of revertants		Approx frequency[b]	
			3 days	6 days	3 days	6 days
XE299-16A	(α *asn1-1 asn2-1*)	3.2×10^8	16	228	5×10^{-8}	7×10^{-7}
XE299-16D	(**a** *asn1-1 asn2-1*)	5.3×10^8	23	120	4×10^{-8}	2×10^{-7}
XE293-8B	(**a** *asn1-2 asn2-2*)	2.4×10^8	2	4	8×10^{-9}	2×10^{-8}
XE293-8D	(α *asn1-2 asn2-2*)	2.4×10^8	0	0	0	0
XE304-2A	(**a** *asn1-3 asn2-3*)	2.1×10^8	1	7	5×10^{-9}	3×10^{-8}
XE304-2B	(α *asn1-3 asn2-3*)	2.0×10^8	3	8	2×10^{-8}	4×10^{-8}

[a] Single colonies of cells were suspended in MV medium supplemented with 10 mM L-asparagine, and these cultures were grown to stationary phase at 30°C (8-ml cultures; final titers approximately 1.3×10^8 to 1.6×10^8 per ml). Cells then were washed three times with distilled water, diluted, and plated onto MV agar plates. After incubation at 30°C for 3 days, colonies were counted. Plates were returned to the incubator and scored again 3 days later. In the table are shown the total number of cells plated (distributed equally among five petri plates for each strain) and the total number of phenotypically revertant colonies scored after the indicated times.

[b] For XE299-16A and -16D, standard deviations were approximately 20 to 50% of means. For all other strains, standard deviations were equal to or greater than the means.

Table 3. They show that frequencies of revertants in recently cloned, haploid mutant populations are low, even though reversion or suppression of either mutant locus would give rise to prototrophic growth. Reversion on the plates or very slow growth of some revertants could account for the increase between 3 and 6 days.

In Table 4 are shown the results of a recon-

struction experiment in which prototrophic cells carrying wild-type alleles of both *ASN1* and *ASN2* or carrying a mutant allele of only one of these genes were plated onto plates of MV medium on which increasing numbers of auxotrophs previously were plated. These experiments show that formation of colonies of prototrophic cells is not inhibited significantly by the presence of up to 9.4×10^7 auxotrophic cells per plate and that mutation in either *ASN1* or *ASN2* alone does not affect this property. Therefore, revertants at either locus should be readily detectable by methods used in these experiments.

Under some conditions, prototrophic revertants were found to be present at a high frequency in populations of diploid cells. Such "revertants" apparently arose by means of mitotic recombination. In Table 5 are summarized the results of an experiment in which this property was quantified. Diploid cells homoallelic or heteroallelic for mutations in *ASN1* and *ASN2* were plated onto minimal medium, and colonies that formed were counted after 2 days of growth. Frequencies of prototrophic cells were very low among auxotrophic populations of cells homoallelic for mutations in *ASN1* and *ASN2*, which confirms that true reversion and reversion due to suppressor mutations occur infrequently. In contrast, prototrophs were present at much higher frequencies in populations of heteroallelic cells, and the frequencies were highly dependent upon the alleles carried by the cells. Similar relative frequencies of prototrophic cells have been measured in other experiments, so the results given in Table 5 do not merely reflect random fluctuations in reversion in the different populations of diploid cells.

Responses of L-asparagine auxotrophs to L-asparagine supplements. In Fig. 1 are presented the results of an experiment in which

TABLE 4. *Recovery of prototrophic cells plated in the presence of* L-*asparagine auxotrophs*[a]

Auxotroph	Auxotrophs/plate	Prototrophs: colonies/plate					
		XE299-1B (**a** *asn1-1 ASN2*)	XE293-1B (**a** *ASN1 asn2-2*)	XE304-1C (**a** *ASN1 ASN2*)	XE299-1C (*α ASN1 asn2-1*)	XE293-1C (*α ASN1 ASN2*)	XE304-1B (*α asn1-3 ASN2*)
XE299-16D (**a** *asn1-1 asn2-1*)	9.4×10^7	54 ± 3	45 ± 4	61 ± 6			
	9.4×10^6	51 ± 10	45 ± 6	48 ± 9			
	9.4×10^5	50 ± 5	45 ± 1	58 ± 5			
	0	47 ± 4	42 ± 1	57 ± 12			
Mean		51 ± 3	44 ± 2	56 ± 6			
XE299-16A (*α asn1-1 asn2-1*)	5.8×10^7				61 ± 4	60 ± 9	51 ± 4
	5.8×10^6				64 ± 12	55 ± 7	60 ± 7
	5.8×10^5				57 ± 7	40 ± 10	46 ± 7
	0				59 ± 2	59 ± 2	41 ± 6
Mean					60 ± 3	54 ± 9	50 ± 8

[a] Indicated numbers of auxotrophic cells were spread onto MV plates, after which approximately 50 prototrophic cells were spread onto the same plates in the combinations given. Plates then were incubated at 30°C, and colonies were counted after 2 days. Each treatment was performed in triplicate. Numbers in the table represent average numbers of colonies per plate ± standard deviation for that treatment. Values are not corrected for revertants arising among the auxotrophs (see Table 3).

TABLE 5. *Frequencies of prototrophic cells in populations of diploid cells homoallelic or heteroallelic for mutations in ASN1 and ASN2*[a]

Diploid no.	Parental strains[b]	Prototrophs/10^7 cells[c]
XE322	XE299-16A (*α asn1-1 asn2-1*)/XE299-16D (**a** *asn1-1 asn2-1*)	0.6 ± 0.6
XE321	XE293-8D (*α asn1-2 asn2-2*)/XE293-8B (**a** *asn1-2 asn2-2*)	0
XE323	XE304-2B (*α asn1-3 asn2-3*)/XE304-2A (**a** *asn1-3 asn2-3*)	1.5 ± 1.7
XE324	XE299-16A (*α asn1-1 asn2-1*)/XE293-8B (**a** *asn1-2 asn2-2*)	172 ± 10
XE326	XE293-8D (*α asn1-2 asn2-2*)/XE299-16D (**a** *asn1-1 asn2-1*)	193 ± 2
XE325	XE304-2B (*α asn1-3 asn2-3*)/XE293-8B (**a** *asn1-2 asn2-2*)	176 ± 21
XE327	XE293-8D (*α asn1-2 asn2-2*)/XE304-2A (**a** *asn1-3 asn2-3*)	146 ± 14
XE328	XE299-16A (*α asn1-1 asn2-1*)/XE304-2A (**a** *asn1-3 asn2-3*)	9 ± 2
XE329	XE304-2B (*α asn1-3 asn2-3*)/XE299-16D (**a** *asn1-1 asn2-1*)	21 ± 8

[a] Cells from recently cloned populations were grown to stationary phase in MV medium containing 10 mM L-asparagine, washed with MV medium, and plated onto MV medium plates in triplicate. Plates were incubated at 30°C, and colonies were counted 2 days later.

[b] Note that two reciprocally constructed diploids were used for each heteroallele combination.

[c] Mean ± standard error of mean.

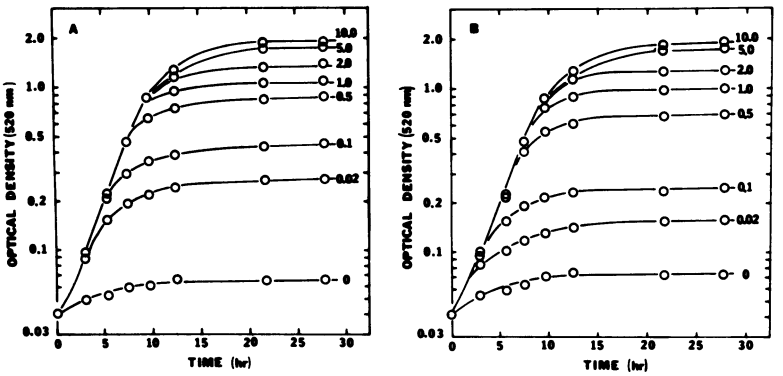

FIG. 1. *(A) Growth of strains XE299-16A (α asn1-1 asn2-1), XE293-8D (α asn1-2 asn2-2), and XE304-2B (α asn1-3 asn2-3) in minimal medium containing 75 mM ammonium ion supplemented with the given concentrations of* L-*asparagine (mM). (B) Growth of the same strains in minimal medium lacking ammonium ion supplemented with the given concentrations of* L-*asparagine (mM). In both experiments, cells were grown overnight to early stationary phase in medium containing 10 mM* L-*asparagine, washed three times with medium containing no* L-*asparagine, diluted into fresh medium containing the appropriate concentrations of* L-*asparagine, and shaken at 200 rpm, 30°C. All three strains behaved identically, and data for all were combined to generate curves shown in the figure.*

auxotrophs were cultured in media supplemented with increasing amounts of L-asparagine. When the initial L-asparagine concentration was 0.1 mM or more, initial doubling time (approximately 2 h) was independent of L-asparagine initial concentration. Similar results were obtained whether or not free ammonium ion (75 mM) was present in addition to L-asparagine. Cells in cultures containing 0.1, 1, or 10 mM L-aspartate behaved identically to those in unsupplemented minimal media (data not shown).

Final cell density in cultures depended strongly upon initial asparagine concentration. When cells were grown in medium with 5 mM or less initial concentration of L-asparagine, less than maximal final cell titers were obtained, even when nitrogen was abundantly available in the form of free ammonium ion. At suboptimal L-asparagine concentrations, cells cultured with L-asparagine as the sole nitrogen source grew to slightly less a culture density than cells in medium containing the same L-asparagine concentration in addition to 75 mM free ammonium ion. The results show that L-asparagine auxotrophs require relatively high concentrations of exogenous L-asparagine and that they can use exogenous L-asparagine not only as an amino acid supplement (see below) but also as an efficiently metabolized supply of nitrogen for cell growth.

Responses of prototrophs carrying *asn1* or *asn2* mutations to ammonium ion supplementation. In Fig. 2, results of an experiment are presented in which haploid cells carrying all possible combinations of *ASN1, asn1-1, ASN2,* and *asn2-1* were cultured in media containing different initial concentrations of free

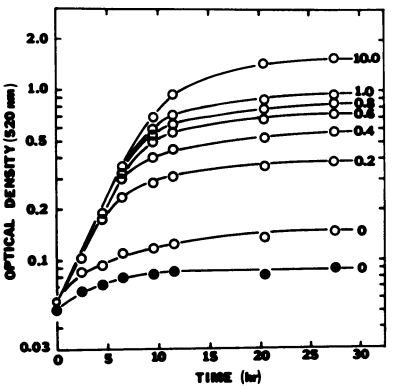

FIG. 2. *Growth of strains carrying all possible combinations of asn1-1, asn2-1, and their respective wild-type alleles in media containing the given concentrations of ammonium ion (mM). Closed circles: XE299-1A (a asn1-1 asn2-1). Open circles: XE299-1B (a asn1-1 ASN2), XE299-1C (α ASN1 asn2-1), and XE299-1D (α ASN1 ASN2) combined. No significant differences were observed among the latter three strains. Cultures were initiated as described in Fig. 1.*

ammonium ion as a nitrogen source. As shown in the figure, prototrophic cells of all three genotypes responded essentially identically. Cells carrying a mutant allele of both *asn1* and *asn2* did not grow in any of the media. These experiments show that growth under limiting nitrogen in the form of free ammonium ion cannot be used to distinguish genotypes of prototrophic cells.

Responses of prototrophs carrying *asn1* or *asn2* mutations to L-asparagine supplements. In Fig. 3 are presented the results of an

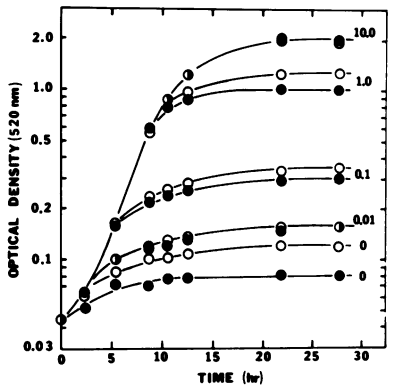

FIG. 3. *Growth of strains carrying all possible combinations of asn1-1, asn2-1, ASN1, and ASN2 in minimal media containing the given initial concentrations (mM) of* L-*asparagine as a sole nitrogen source. Closed circles: XE299-1A (a asn1-1 asn2-1). Open circles: XE299-1B (a asn1-1 ASN2), XE299-1C (α ASN1 asn2-1), and XE299-1D (α ASN1 ASN2) combined. No significant differences were observed among the latter three strains. Cultures were initiated as described in Fig. 1.*

experiment in which haploid cells carrying all four combinations of *ASN1, asn1-1, ASN2,* and *asn2-1* were cultured in media containing different initial concentrations of L-asparagine as sole nitrogen source. Again, prototrophic cells of all three geneotypes behaved identically. Auxotrophs carrying both *asn1-1* and *asn2-1* grew to a final titer that was only slightly (perhaps insignificantly) less than the titers achieved by prototrophs at any given initial concentration of the amino acid.

Lack of gene dosage effect on growth response of diploid cells. Diploid cells carrying various combinations of *ASN1, asn1-1, ASN2,* and *asn2-1* were tested for ability to grow in minimal medium (MV) or in minimal medium supplemented with 10 mM L-asparagine. All diploids except those that were homozygous for *asn1-1* and *asn2-1* grew about equally well in MV or in MV supplemented with the amino acid (data not presented). In unsupplemented minimal medium, doubling times of cultures of cells containing only a single wild-type allele of just one of these genes (average, 2.2 h) were not greatly different from doubling times of cultures of cells containing two, three, or even four wild-type alleles at these loci (average doubling time = 1.8 h). In medium in which L-asparagine (10 mM) was present, all diploids (including those that were homozygous for *asn1-1* and *asn2-1*) grew with nearly equal doubling times of about 1.8 h. In each medium, when cells grew, final cell titers were about the same for cells of all genotypes.

Requirement for L-asparagine. Auxanographic analyses showed that none of the other 19 common L-amino acids singly or in combination could substitute for L-asparagine as a growth promoter for any of the auxotrophs. More detailed studies were performed with amino acids that can serve as sole nitrogen sources for growth to exclude the possibility that auxotrophs grow in medium supplemented with L-asparagine for the trivial reason that it is an alternative nitrogen source present in the medium. Wild-type and auxotrophic cells were grown in MV medium lacking ammonium ion (MV-NH_3) supplemented with one of a variety of other alternative sources. In all cases tested, the L-asparagine auxotroph could grow in the presence of these nitrogen sources only if L-asparagine also was present (Table 6). Thus, it seems unlikely that L-asparagine is serving merely as a surrogate nitrogen source for general metabolism in the auxotroph. However, these experiments do not exclude the possibility that L-asparagine is necessary in the auxotrophs for functions in addition to its use as a precursor for protein synthesis.

DISCUSSION

Data presented in this paper clearly show that L-asparagine auxotrophy in *S. cerevisiae* is the

TABLE 6. *Doubling times of cultures of wild-type and* L-*asparagine auxotrophic cells in media containing different nitrogen sources*[a]

Nitrogen source[b]	MV-NH_3[c]		MV-NH_3 + L-ASN[d]	
	S288C	XE256-1D	S288C	XE256-1D
None	–	–[e]	2.3	2.3
NH_4^+	2.4	–	2.0	2.1
D-Asparagine	4.1	–	2.1	2.0
L-Aspartate	2.0	–	2.0	2.1
L-Glutamine	2.0	–	2.0	2.0
L-Glutamate	2.3	–	2.0	2.0
L-Arginine	2.1	–	2.1	2.0
L-Proline	3.9	–	2.2	2.2

[a] Strain S288C is wild type for L-asparagine requirement. Strain XE256-1D (*α asn1-1 asn2-1*) requires L-asparagine for growth. Doubling times are the averages of duplicate determinations (in hours) and are reproducible to within about 0.2 h.

[b] Media contained one of these nitrogen sources at 10 mM concentration. All media were adjusted to pH 5.0 ± 0.2 before being filter sterilized.

[c] Cells were cultured in minimal medium in which the given nitrogen source was the only available supply of nitrogen.

[d] Cells were cultured in medium containing L-asparagine (10 mM) in addition to the given nitrogen source.

[e] In these cultures, the cell number approximately doubled, after which no further growth occurred.

result of mutation in each of two unlinked cistrons, *ASN1* and *ASN2*. That two apparently independent mutations are required to engender auxotrophy accounts for previous failure to isolate such mutants without applying enrichment procedures. The situation in yeast is in distinct contrast with the case in *Escherichia coli* and cultured Chinese hamster cells, in which mutation in only a single known cistron can cause a requirement for the amino acid (2, 5). In haploid yeast cells, mutation in only one of the two cistrons yields growth indistinguishable from that of wild-type cells under all conditions used in the present experiments, even when growth is severely limited by nitrogen available only in the form of L-asparagine. In diploid cells, the presence of only a single wild-type allele of either cistron is sufficient to allow prototrophic growth. Haploid auxotrophs are highly stable genetically, but certain heteroallelic diploid populations rapidly accumulate prototrophs, presumably by mitotic recombination. Care must be taken to ensure the genetic integrity of such populations.

At this time, no way has been found to recognize directly a haploid cell that carries a mutation in only one of the two cistrons. However, by observing the segregation pattern of auxotrophy and prototrophy among the progeny of a cross of such a putative cell to a cell known to be carrying both mutations, it can be inferred that the parent cell was carrying a mutation in *ASN1* or *ASN2*. Which cistron bears the mutation in such a cell can be inferred by performing another series of crosses of the test cell to cells known to be carrying a mutation in one of the cistrons.

The genetic data presented in this report suggest that two independent pathways might be involved in L-asparagine synthesis in this species. If so, either pathway alone provides sufficient L-asparagine for maximal growth. Another possibility is that only a single synthetic pathway exists, mutation in a regulatory gene accounting for the second locus' effects. It is also possible that *ASN1* and *ASN2* represent the outcome of a gene duplication event that occurred during the evolution of this species. Biochemical and physiological consequences of mutation in *ASN1* and *ASN2* are now being determined.

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