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Mutants of *Saccharomycopsis lipolytica* Defective in Lysine Catabolism

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Wild-type strains of *Saccharomycopsis lipolytica* are able to use lysine as a carbon or a nitrogen source, but not as a unique source for both. Mutants were selected that could not use lysine either as a nitrogen or as a carbon source. Some of them, however, utilized *N*-6-acetyllysine or 5-aminovaleric acid. Many of the mutants appeared to be blocked in both utilizations, suggesting a unique pathway for lysine degradation (either as a carbon or as a nitrogen source). Genetic characterization of these mutants was achieved by complementation and recombination tests.

Lysine catabolism in eukaryotic cells has been studied in animals, plants, and filamentous fungi, but few investigations have been done in yeasts. Two main pathways, which may not be exclusive, seem to occur. One is the saccharopine-aminoadipic acid pathway, a reversal of the biosynthetic pathway found in yeasts and fungi (4, 13, 17). It involves the removal of the terminal amino group. The second pathway, or pipecolic pathway, involves the removal of the α-amino group. It exists in animals (23), in plants (5, 8, 11), and in filamentous fungi (15, 26). Interconversion of pipecolate and aminoadipic acid has been reported in most of these systems (2, 22), so both of the catabolic pathways presumably end in the same metabolite (aminoadipic acid), which is subsequently transformed into glutarate and could serve as carbon source. The duplication of the lysine degradation pathways was shown repeatedly to be related to the ability to catabolize both L and D forms of this amino acid, each of them having its more or less specific pathway (9, 10, 16; F. P. Guenguerich, Ph.D. thesis, Vanderbilt Univ., Nashville, Tenn., 1973).

In yeasts, the situation is more complex, and various patterns can be found. For example, *Saccharomyces cerevisiae* can use lysine as a carbon source, but not as a nitrogen source, whereas other yeast species can use lysine as a nitrogen but not a carbon source (1, 12, 25, 29). Up to now, no satisfactory explanation of this phenomenon has been proposed.

A few studies with labeled precursors have been conducted. The so-called acetylated compound pathway was proposed by Rothstein for *Hansenula saturnus* (21): the terminal amino group of lysine is first blocked by acetylation, and deamination then occurs, leading to 2-hydroxy-6-acetyl-aminocaproic (2-hydroxy-*N*-6-acetyllysine) or, alternatively, to 5-aminovaleric acid. Sagisaka and Shimura (24) have studied the metabolic fate of [6-^{14}C]aminoadipic acid in *Torulopsis utilis*: they proposed a pathway via 2-ceto-6-aminocaproate and 5-aminovalerate or 2-hydroxylysine.

To study the degradation of lysine, we have chosen the yeast *Saccharomycopsis lipolytica*, which has the unusual property of being able to utilize lysine as a carbon as well as a nitrogen source.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. They have all been obtained in the laboratory as derivatives of two wild-type strains of opposite mating type, W29 and Z30 (6). Double mutants were isolated as meiotic recombinants, after crossing the two haploids, and their genotype was checked by complementation tests. Culture media employed in maintenance, as well as growth and mating assays, have been described (6). In synthetic medium (mineral) the carbon source is supplied by 10 g of glucose per liter or 15 g of amino acids per liter; nitrogen source by $(NH_4)_2SO_4$ (5 g/liter [N 5,000 medium] or 100 mg/liter [N 100 medium]), or by 1 g of lysine per liter. These media are eventually supplemented with amino acids or nucleotides at a concentration of 100 mg/liter.

Growth assays. To determine the effect of a particular compound on the generation time or the yield of a given strain, the growth in liquid cultures was observed. Erlenmeyer flasks (100 ml, containing 20 ml of medium) were inoculated (about 10^6 cells/ml) and incubated at 30 C on a shaker. Growth was recorded either by counting the cells (Malassez counting chamber) or by measuring the turbidity of the culture (nephelometer).

Assimilation tests were achieved by depositing

TABLE 1. *List of yeast strains used*

Designation	Relevant genotypes	Characteristics	Mating type
W29		Wild-type strain	A
mg-5[a]	*lys1.5*	Transdehydrolysine-resistant isolate of W29	A
8041-2[b]	*his1*	Histidine requiring	A
8041-3[b]	*his1*	Histidine requiring	A
105-2	*ura1*	Uracil requiring	B
9901-9[c]	*ade1, SU3, lys1.1*	Adenine requiring	B
9901-10[c]	*ade1, SU3, lys1.1*	Adenine requiring	B
8701-12[b]	*ade1*	Adenine requiring	A
8701-13[b]	*ade1*	Adenine requiring	B
4304-4	*lys2.14*	Lysine requiring[d]	A
8041-6	*lys1.13*	Lysine requiring[e]	B
9203-1	*lys1.5, lys 2.12, ade1*	Lysine and adenine requiring[d]	B

[a] mg-5 is resistant to 10^{-4} M 4,5-transdehydrolysine and accumulates L-lysine (7). *Lys1.5* is allelic to *lys1.13* and *lys1.1*

[b] Standard strains.

[c] *SU3* is a dominant suppressor of *lys1.1*, unlinked to *lys1.1*.

[d] 2-Aminoadipic acid accumulating.

[e] Lysine can be replaced by 2-aminoadipic acid.

drops (about 10^4 to 10^5 cells/ml) of the culture to be tested on solid selective media. Growth was recorded after 4 days. For carbon or nitrogen source assimilation tests, a control was run without a carbon or without a nitrogen source, since there is always slight growth on agar alone, even with starved cells.

Cell-free extracts and pool determination. Cells were obtained from 100-ml cultures in liquid mineral medium and harvested at a given fixed optical density (usually in log phase between 6×10^7 and 7×10^7 cells/ml).

After three washings with ice-cold water, 300 mg (wet weight) of cells was resuspended in 5 ml of water. Two milliliters of this suspension was filtered on a Whatman glass-fiber filter GFA and used for dry-weight estimation. The remaining suspension was boiled for 20 min to extract the pool of free amino acids. After centrifuging cellular debris, the clear supernatant was filtered on a membrane filter (Millipore) and used for pool estimation.

The specific determination of lysine was achieved by the method of Shimura and Vogel (27; Fed. Proc. **20:**10, 1961), slightly modified to increase its specificity. To 0.3 ml of the pool preparation to be tested are added successively: 0.2 ml of 3 N HCl and 0.5 ml of ninhydrin (15% in methylcellosolve). After incubation in boiling water for 1 h in sealed tubes, the preparation is cooled and 1 ml of H_3PO_4 (diluted 1:5) is added. A red color develops and the optical density is read at 450 nm against a blank treated in the same way, but without extract. Concentrations are determined with standards of known lysine concentrations, between 5×10^{-5} and 5×10^{-4} M. Pool concentrations are thus expressed as micromoles of lysine per gram of dry weight.

Chromatographic procedures. The composition of the pools extracted was qualitatively estimated by chromatography with commercial standards, in three different solvent systems (system 1: chloroform-methanol-ammonia [2:2:1]; system 2: butanol-acetic acid-water [4:1:5]; system 3: phenol-water [15:2 wt/vol]). R_f values of commercial products in these three systems were, respectively: L-lysine: 0.26, 0.08, 0.06; *N*-6-acetyllysine: 0.78, 0.18, 0.50; and 5-aminovalerate: 0.53, 0.32, 0.33.

Chromatography was performed on Silica gel G (Merck Fertigplatte or Eastman chromatogram), usually for 2 h. Chromatograms were revealed either by spraying a ninhydrin solution (in methylcellosolve), or by autoradiography when labeled compounds were used.

Autoradiography was performed according to the method of Randerath (20). The chromatogram was pressed against an X-ray film (Kodak RPS Mat) between two glass plates, and the sandwich was wrapped in an aluminum sheet. Exposure was carried out for 5 days at 25 C, and the film was then developed (10 min in Kodak X-ray developer, 5 min in Kodak X-ray fixer). The labeled spots could then be cut off the chromatogram and counted in a liquid scintillation counter.

Mutant isolation. Mutants defective in the assimilation of a given carbon or nitrogen source were obtained after ultraviolet treatment and nystatin enrichment (6) on a medium where the defective mutants could not grow (e.g., a minimal medium containing 15 g of lysine per liter instead of glucose for isolating mutants affected in the utilization of lysine as carbon source).

Treated suspensions were diluted serially and plated on nonselective medium, and, after 4 days, the resulting colonies were replica plated on selective media. Presumptive mutants were purified by streaking on complete agar medium and tested in drops for the assimilation of putative lysine catabolic products.

Only one mutant from each clone mutagenized was saved as a precaution against reisolation of the same mutant. Mutants are recorded as $lysC^+/lysC^-$, $lysN^+/lysN^-$, when they grow (or not) on lysine as carbon or nitrogen source. The same convention is valid for mutants using *N*-6-acetyllysine or 5-aminovalerate (a $6nalC^-$ $avaN^+$ strain grows on 5-aminovalerate as a nitrogen source but not on *N*-6-acetyllysine as a carbon source).

Genetic tests. Complementation tests in *S. lipolytica* are usually conducted by mixing parents with complementary auxotrophic markers on minimal medium (6). In some cases, however, mutants defective in lysine catabolism (hereafter referred to as lyc^-), were obtained in nonauxotrophic strains. These were first crossed to reference strains (adenine or histidine requiring), and the resulting diploids were isolated on minimal medium with lysine as a unique carbon source. After sporulation of these diploids, the auxotrophic lyc^- double mutants could be obtained.

The complementation tests were then conducted as follows. Two compatible lyc$^-$ mutants carrying complementary auxotrophic requirements were crossed, and the diploids were isolated on minimal glucose ammonia medium. These diploids were then tested on lysine as a carbon source to determine their lyc$^+$ or lyc$^-$ phenotype.

The meiotic segregations were generally studied by plating randomly mixed ascospores in paraffin oil as previously described (6). Spores were plated on complete yeast extract medium, and the resulting colonies were replica plated onto selective media (e.g., lysine as carbon source, or 5-aminovalerate as nitrogen source.) Replica plates were read after 4 days, except for the capacity to catabolize lysine (1 week). In some cases, asci were dissected with a micromanipulator, according to the procedure of Bassel et al. (3).

Chemicals. [^{14}C]lysine (random labeling) was a gift of the Commissariat à l'Energie Atomique and 4,5-transdehydrolysine was synthesized on request by the École de Chimie de Mulhouse. Other products are commercially available. Solvents are of the highest grade of purity available.

RESULTS

Utilization of lysine and its putative catabolic products. The growth responses of our strains on compounds tested are listed in Table 2.

The wild-type W29 can use lysine as a carbon or nitrogen source. With lysine as the carbon source, the generation time was about 2.5 to 3 h (versus 2 h on glucose), but glucose-grown cells showed a very long lag when transferred to lysine as a carbon source. This was particularly demonstrable with solid medium (2 days lag).

Among the intermediates tested, only *N*-6-acetyllysine or 5-aminovalerate was able to mimic lysine as a potential source of carbon or nitrogen. They were used much faster than lysine as a carbon source. Pipecolic acid and 2-aminoadipic acid served as a nitrogen source only, but growth was rather poor. D-Lysine was not used.

TABLE 2. *Growth responses of wild-type strains on compounds tested as carbon or nitrogen sources*[a]

Compound tested	C source (15 g/liter)	N source (1 g/liter)	Both (15 g/liter)
L-Lysine	+	+	−
N-6-acetyllysine	+	+	±
5-Aminovaleric acid	+	+	+
6-Aminocaproic acid	−	−	
Pipecolic acid	−	±	
2-Aminoadipic acid	−	±	
Saccharopine	−	−	
D-Lysine	−	−	
Arginine	+	+	
N-2-acetyllysine	−	±	
5-Hydroxylysine	−		

[a] Growth responses: +, growth similar to that on glucose or ammonium medium; −, growth not better than on a medium lacking carbon or nitrogen source; ±, intermediate response.

Lysine could not serve simultaneously as a source of both carbon and nitrogen. However, some compounds related to lysine could. 5-Aminovalerate was used in this way (generation time about 6 h), and *N*-6-acetyllysine gave intermediate responses (generation time, about 14 h). Cells growing on lysine as a carbon source or on a high level of external lysine (10 g/liter) in a glucose medium accumulated *N*-6-acetyllysine. 5-Aminovalerate was not detected by the chromatographic procedures used.

The origin of the accumulated *N*-6-acetyllysine was further confirmed by labeling experiment. If radioactive lysine (^{14}C, randomly labeled) was added 1 h before extracting the pool of a 24-h-old culture grown on glucose-ammonium medium supplemented with 15 g of L-lysine per liter, the presence of radioactive *N*-6-acetyllysine was clearly demonstrated by chromatography and autoradiography. No other spots are labeled under these conditions; for instance, 5-aminovaleric acid was never detected, indicating that it is not accumulated.

Phenotypic tests on mutants defective in lysine catabolism. Mutants were isolated as lysC$^-$, lysN$^-$, or avaN$^-$ (see above). Their phenotypes and the mode of selection are shown in Table 3. According to their growth responses on different carbon sources, they can be ordered as shown on Fig. 1. The inability of class 2 mutants to use lysine and *N*-6-acetyllysine as a nitrogen source is explicable by our scheme of lysine degradation, but that of the class 3 mutants is not. The inability of class 3 mutants to grow on lysine as a sole nitrogen source will be discussed below.

2-Aminoadipic acid is a poor nitrogen source in lysN$^-$ strains. The possibility that this compound is normally first converted into lysine by the lysine anabolic pathway and then eventually used as a nitrogen source is discussed below. The same is true for pipecolic acid.

The reason for checking 4,5-transdehydrolysine resistance and the ability to utilize arginine as a carbon source relies on preliminary observations of the uptake of lysine by *S. lipolytica* (J. M. Beckerich, personal communication). These compounds strongly inhibit lysine uptake and are probably transported by the same permease system as lysine in this yeast. So, the facts that (i) all mutants so far tested are sensitive to the lysine analogue (if they do not carry *lys1.5*) and (ii) they grow on arginine as a carbon source make it unlikely that the inabil-

TABLE 3. *Phenotypic classes among lyc$^-$ mutants*

Phenotypic class	Isolate no.	Original strain	Selected as	Carbon source				Nitrogen source				Growth on
				Lys	6nal[a]	ava	Arg	Lys	6nal[b]	ava	aaa[c]	TDL[d]
1	*lyc5* *lyc10* *lyc33*	W29 W29 8041-3	lysC$^-$ lysC$^-$ lysN$^-$	−	+	+	+	−	+	+	−	−
2	*lyc38* *lyc40* *lyc42*	105-2 105-2 105-2	lysN$^-$ lysN$^-$ lysN$^-$	−	−	+	+	−	−	+	−	−
3	*lyc1* *lyc18* *lyc22* *lyc32*	W29 mg-5 8041-2 8041-3	lysC$^-$ lysC$^-$ lysN$^-$ lysN$^-$	−	−	+	+	−	+	+	−	−
4	*lyc43* *lyc44*	W29 mg-5	avaN$^-$ avaN$^-$	−	−	−	+	+	+	−	+	−
5	*lyc14* *lyc15* *lyc16*	mg-5 mg-5 mg-5	lysC$^-$ lysC$^-$ lysC$^-$	−	−	−	+	+	+	+	+	

[a] 6nal, *N*-6 acetyllysine; ava, 5-aminovalerate; aaa, 2-aminoadipidic acid; TDL, 4,5-transdehydrolysine.

[b] 2-nal gave the same results as lysine.

[c] Pipecolic acid gave same results as 2-aminoadipic acid.

[d] Resistance to 4,5-transdehydrolysine, 5×10^{-5} M, was not tested when strains were carrying *lys1.5*.

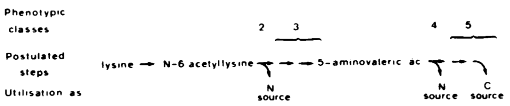

FIG. 1. *Proposed arrangement of lyc$^-$ mutants along lysine catabolic pathway. Mutants are grouped into several classes, according to their growth responses to lysine, N-6-acetyllysine, and 5-aminovaleric acid tested as carbon or nitrogen sources. The pathway is suggested by the growth tests and mutant responses.*

ity to use lysine as carbon source is due to an alteration in uptake capacities.

This was of particular importance in the case of class 1 mutants, which grow normally on all postulated intermediates, but not on lysine. As pointed out by Surdin et al. (28), mutants can be obtained that may have partially lost the ability to transport a given amino acid; in this case, however, a higher level of external supply is required to cover a particular need. Thus we tried to construct a double mutant that carried both the mutation under investigation and lysine auxotrophy. Several presumptive catabolic mutants were crossed with 9901-9 and 9901-10 (*B*, *ade1*, *SU3*, *lys1.1*) and the progeny were analyzed, as shown on Table 4.

There was no inviable class, and the double mutants (*su3*$^{+}$, *lys1.1*, *lyc*$^{-}$) were grown on limiting amounts of lysine. They showed the same linear response to external supply of lysine as (*su3*$^{+}$, *lys1.1*, *lyc*$^{+}$) between concentrations of 5×10^{-5} M and 7.5×10^{-4} M (yield versus lysine supplied).

Finally, mutants of classes 1, 2, and 3, which cannot use lysine either as carbon or as nitrogen sources, accumulate much more lysine when grown in the presence of this amino acid than does, for example, the W29 strain (Fig. 2). But, as shown by autoradiography, there is no accumulation of *N*-6-acetyllysine.

Complementation and linkage among lyc$^-$ mutants. In each class, at least one mutant was shown to be the result of a single mutation by crossing it with a standard strain. In all cases, lyc$^-$ was recessive and segregated as a single gene. Auxotrophic lyc$^-$ strains under both mating types were isolated.

Complementation tests were carried among some of them as shown on Table 5. Complementation groups for the various phenotypic classes are given on Table 6. Each diploid, first isolated as a prototroph, was tested on ammonium medium without glucose, containing 15 g of lysine per liter. Eight complementation groups were defined in this way. Linkage studies were confined to class 1, 2, and 3 mutants. There is apparently no physical association between the different loci (Table 7).

Role of 2-aminoadipate and pipecolate in lysine catabolism. In *S. cerevisiae*, 2-aminoadipate is used as a nitrogen source,

TABLE 4. *Existence of lysine-less, lyc$^-$ mutants in the progeny of a (lys$^-$/lyc$^-$) diploid*[a]

Pheno-typic class of lyc$^-$ used	Crosses (genotypes)	Phenotypes observed				Total no. of:	
		lys^{-b} lyc$^+$	lys$^-$ lyc$^-$	lys$^+$ lyc$^-$	lys$^+$ lyc$^+$	lyc$^-$	lyc$^+$
1	*lyc10* × *lys1.1, SU3, ade1*	11	9	49	43	58	54
2	*lyc5* × *lys1.1 SU3, ade1*	28	26	74	76	100	104
3	*lyc1* × *lys1.1, SU3, ade1*	18	15	46	38	61	56
3	*lyc18* × *lys1.1, SU3, ade1*	13	10	40	35	50	48
5	*lyc15* × *lys1.1, SU3, ade1*	38	37	98	104	135	142

[a] Tester strain was 9901.10 except for cross with lyc18, where 9901.9 was used. For simplicity, the segregation of *ade1* gene is not recorded.

[b] lys$^-$ clones are all of (*lys1.1*, *su3*$^+$) genotype, whereas lys$^+$ clones can be (*lys1.1*, *SU3*), (+, *SU3*) and (+, *SU3*$^+$); one thus expects about 3 lys$^+$:1 lys$^-$.

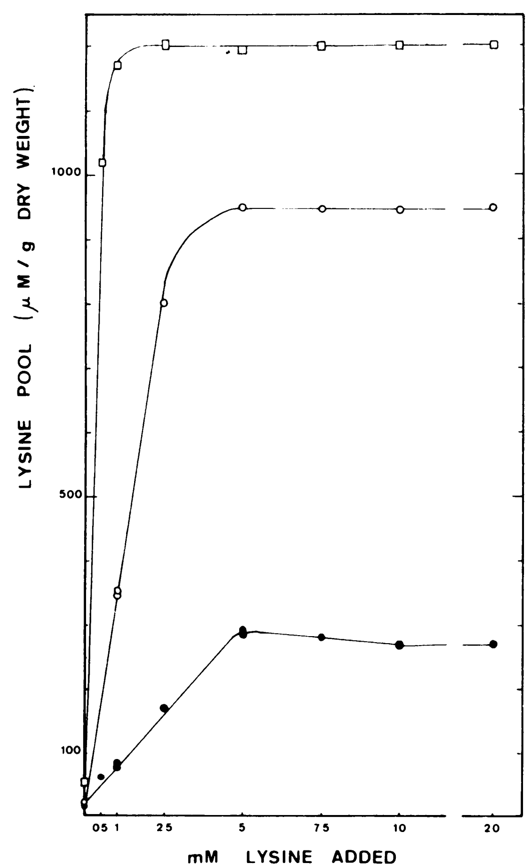

FIG. 2. *Variations of the free intracellular lysine content of cells grown on N 5,000 medium supplemented with various amounts of lysine. Cells were harvested in exponential phase at about 7.5 cells/ml and subjected to pool analysis (see text). The lysine pool content is plotted against the initial amount of lysine added to the medium. Symbols: (●), W29 wild-type strain; (○), lyc1 mutant; (□), lyc5 mutant.*

whereas lysine is not (14). Results are ambiguous with *S. lipolytica*, where the wild-type strain grows poorly when the nitrogen source is supplied by 2-aminoadipate or pipecolate. Both lysine-less mutants blocked after 2-aminoadipate in the anabolic pathway and lysC$^-$ lysN$^-$ mutants grow even less or not at all under these conditions, but lysC$^-$ lysN$^+$ mutants (such as *lyc44*) grow rather well; they provide better material for studying whether the conversion of these compounds into lysine is necessary for their utilization as a nitrogen source.

To investigate this, we developed strains carrying the *lyc44* defect and a mutation in the anabolic pathway after 2-aminoadipate (e.g., *lys2.12* or *lys2.14*). As a reference, we used strains of (*lys1.13*, *lyc44*) genotype, *lys1.13* affecting the pathway at its beginning, and the same combinations of auxotrophic markers with *lyc1*, *lyc5*, which both prevent the utilization of lysine as a nitrogen source in the wild-type strain. Results of the growth tests are listed in Table 8. It appears that all mutations or groups of mutations that affect either the conversion of 2-aminoadipate into lysine (like *lys2.12*, *lys-2.14*), or the conversion of lysine into *N*-6-acetyllysine (like *lyc1* or *lyc5*), affect the utilization of 2-aminoadipate and pipecolate as nitrogen source.

The aaaN$^-$ phenotype of strains carrying a mutation that leads to accumulation of 2-aminoadipate could be due to a limited uptake of this compound. It was therefore verified that (*lys1.13*, *lys2.12*) strains do not accumulate 2-aminoadipate when cultivated on limiting amounts of lysine, but that this amino acid was indeed accumulated when the medium was supplemented with 1 g of 2-aminoadipate per liter.

Studies on class 3 mutants. All mutants of

TABLE 5. *Complementation among lyc⁻ mutants*

Classes	Class 1			Class 2			Class 3				Class 4		Class 5	
	5	10	33	38	40	42	1	18	22	32	43	44	14	15
Class 5														
16	+			+									+	−
15	+	+		+	+		+	+	+	+	+	+	+	
14	+			+			+	+	+	+	+	+		
Class 4														
44		+		+			+	+	+	+	−			
43		+		+			+	+	+	+				
Class 3														
32	+	+	+	+[a]	+	+	+	+	−					
22	+	+	+	+	+	+	+	+						
18	+	+	+	+	+	+	+							
1	+	+	+	+	+	+								
Class 2														
42	+	+	+	−	−									
40	+	+	+	−										
38	+	+	+											
Class 1														
33	−	−												
10	−													

[a] All diploids obtained between class 2 and class 3 mutants grew poorly.

TABLE 6. *Complementation groups*

Phenotypic classes	Complementation groups
5	*lyc14*
5	*lyc15, lyc16*
4	*lyc44, lyc45*
3	*lyc1*
3	*lyc18*
3	*lyc22, lyc32*
2	*lyc38, lyc40, lyc42*
1	*lyc5, lyc10, lyc33*

TABLE 7. *Meiotic segregations of lyc⁻/lyc⁻ diploids*[a]

Strains	Classes	lyc⁺ spores	Total no. of spores tested	lysC⁺/ total (%)
lyc5/lyc10	1 × 1	0	412	ND[b]
lyc5/lyc33	1 × 1	0	375	ND
lyc5/lyc40	1 × 2	86	307	28
lyc38/lyc40	2 × 2	0	316	ND
lyc5/lyc18	1 × 3	39	165	24
lyc10/lyc1	1 × 3	31	90	34
lyc38/lyc22	2 × 3	145	563	26
lyc38/lyc18	2 × 3	55	247	33
lyc38/lyc1	2 × 3	97	402	24
lyc15/lyc10	5 × 1	82	246	33
lyc15/lyc1	5 × 3	36	160	23
lyc1/lyc18	3 × 3	48	187	26
lyc18/lyc22	3 × 3	56	209	27
lyc1/lyc32	3 × 3	28	111	25
lyc22/lyc32	3 × 3	0	452	ND

[a] The progeny were studied by mass isolation of the spores, as described, on minimal medium supplemented for all auxotrophic requirements and were replica-plated onto minimal medium containing lysine as a carbon source and supplemented as described.

[b] ND, Not determined.

this class, which are distributed among at least three different loci, did not use lysine as a nitrogen souce, but grew well on a medium where *N*-6-acetyllysine was substituted for ammonium. From their responses on different carbon sources, they have been located before 5-aminovaleric acid (Fig. 1), so they should convert lysine into *N*-6-acetyllysine and use it in the same way they use externally supplied *N*-6-acetyllysine. Since these mutants do not genetically lack the activities involved in the conversion of lysine (as they complement with class 1 mutants), the possibility remains that the step missing is either not activated or not induced in class 3 mutants.

To test the possibility that the expected "activator" might be a product of the catabolic pathway not formed in these mutants, we tried

TABLE 8. *Use of 2-aminoadipate, pipecolate, and lysine as nitrogen sources by lysine-less, lyc⁻ mutants*

Strains tested (genotypes)	Growth responses on nitrogen source[a]		
	2-Amino-adipate	Pipe-colate	Lysine
lys1.13	±	±	+
lys2.14	−	−	+
lys2.12	−	−	+
lyc5	−	−	−
lyc44	+	+	+
lys1.13, *lyc5*	−	−	−
lys1.13, *lyc44*	+	+	+
lys2.14, *lyc1*	−	−	−
lys2.14, *lyc44*	−	−	+
lys1.13, *lys2.12*, *lyc1*	−	−	−
lys1.13, *lys2.12*, *lyc44*	−	−	+

[a] All media supplemented with 50 mg of L-lysine per liter and 50 mg of adenine per liter to compensate for auxotrophic requirements.

TABLE 9. *Growth responses of the progeny of a (lyc1+/+lyc44) diploid*

No. of tetrads	Phenotypes of spores				Inferred genotypes	
	lysC	lysN	avaN	lysN[a]		
7	−	−	+	−	*lyc1*	+
	−	−	+	−	*lyc1*	+
	−	+	−	+	+	*lyc44*
	−	+	−	+	+	*lyc44*
2	−	−	−	+	*lyc1*	*lyc44*
	−	−	−	+	*lyc1*	*lyc44*
	+	+	+	+	+	+
	+	+	+	+	+	+
5	−	−	−	+	*lyc1*	*lyc44*
	−	−	+	−	*lyc1*	+
	−	+	−	+	+	*lyc44*
	+	+	+	+	+	+

[a] 5-Aminovalerate added, 10 mg/liter.

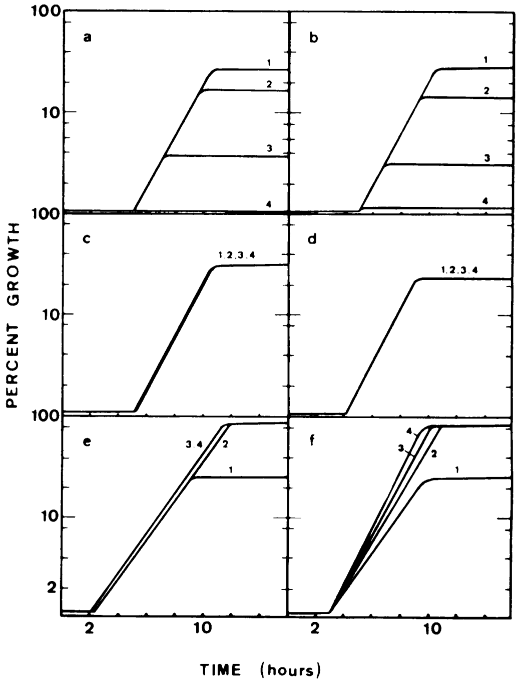

FIG. 3. *Growth responses on N 100 medium supplemented with various concentrations of lysine of the wild-type strain W29 (f) and of several lyc⁻ mutants: lyc5 (a), lyc33 (b), lyc1 (c), lyc18 (d), and lyc44 (e). Strains were starved for 14 h on a glucose medium lacking nitrogen source and transferred to N 100 medium containing various amounts of lysine: 1, without lysine; 2, 100 mg/liter; 3, 1 g/liter; 4, 5 g/liter. Responses of lyc1 and lyc18 are identical under these conditions.*

to test lysine utilization in the presence of 5-aminovaleric acid. But as these mutants responded poorly to 5-aminovalerate concentrations below 100 mg/liter (sufficient to allow a certain growth as nitrogen source), we tested the possibility in the double mutant *lyc1*, *lyc44* (phenotype, avaN⁻). Growth responses of the progeny of a cross involving *lyc1* and *lyc44* are given in Table 9. The recombinant strains (*lyc1*, *lyc44*) grew on lysine as nitrogen source, but only in the presence of 10 mg of 5-aminovaleric acid per liter.

A possible explanation for the lack of growth of *lyc1* on a medium containing lysine as a nitrogen source and supplemented with 5-aminovalerate is that this compound cannot be accumulated in amounts sufficient to induce (or activate) the first step, and that its catabolism does not liberate enough nitrogen to allow growth.

Studies on class 1 mutants. All catabolic mutants were tested for lysine utilization in ammonium-rich (N 5,000) and in ammonium-limiting (N 100) medium. Except for class 1 mutants, there was no decrease in the growth rate or in the final yield with increasing lysine concentrations. Moreover, the final yield, in ammonium-limiting medium, was significantly increased for the mutants able to use lysine as a nitrogen source (Fig. 3). As no diauxic growth occurred under these conditions, it is suspected that utilization of lysine as a nitrogen source is not affected by ammonium.

On the contrary, class 1 mutants were strongly affected by lysine, not in their initial growth rate, but in the final yield. In this case, lysine seems to prevent ammonium utilization, and, moreover, to cause vegetative death under these conditions: the plating efficiency (cells plated in complete medium versus cells counted) of 10-h-old cultures decreased with increasing lysine concentration (55, 53, 5, 2, and 0.5% for cultures supplemented, respectively, with lysine concentrations of 0, 0.1, 1, 5, and 10 g/liter, 1 g/liter, 5 g/liter, and 10 g/liter).

As class 1 mutants are monogenic, the same mutational event seems to be implicated in the inability to use lysine as carbon source on N 5,000 medium (where no toxicity was apparent) and in the lysine sensitivity in ammonium-limiting medium. Both characters appear to be recessive in diploids. The sensitivity was very specific to lysine, since no other amino acid could mimic lysine.

DISCUSSION

If we assume that lysine degradation in *S. lipolytica* effectively occurs via *N*-6-acetyllysine and 5-aminovaleric acid, the following tentative scheme can be discussed (see Fig. 4).

The conversion of lysine into *N*-6-acetyllysine has been described in a number of different systems (10, 19, 21, 26). This step seems to be irreversible in vivo, in *S. lipolytica*, since lysineless strains cannot use *N*-6-acetyllysine instead of lysine. Acetylation of the terminal amino group could play a double role. First, it would prevent ϵ deamination, and therefore exclude pipecolic and aminoadipic acids from the "normal" pathway. This prediction agrees with our results. Second, it would facilitate removal of the α amino group: it has been shown that lysine is the only amino acid that does not undergo reversible deamination in the α position. However, if the terminal amino position is substituted, the α amino group can be removed (18).

The second step of the proposed pathway leads to 2-keto-*N*-6-acetamidocaproic acid; class 2 mutants are probably affected in a specific transaminase. It should be emphasized here that our scheme is based on the presumption that there is a unique pathway for the utilization of lysine as a carbon and as a nitrogen source. Class 2 mutants are unable to use lysine and *N*-6-acetyllysine as a nitrogen source; they fail also to use lysine as a carbon source.

Deacetylation of 2-keto-*N*-6-acetamidocaproate by an ϵ-lysine acylase has been shown to occur in mammals (18); it yields pipecolic acid by subsequent cyclization of the product formed. The occurrence of such an activity in *S. lipolytica* remains questionable, since we have shown that externally added pipecolic acid has to be first converted into lysine in order to be used as a nitrogen source. This does not rule out the possibility that internally formed 2-keto-*N*-6-acetamidocaproate is deacetylated to pipecolic acid, which may be a side product of the breakdown of lysine. Our results favor the hypothesis that pipecolic acid may then be recycled into lysine by the anabolic pathway, as has been shown in other systems (26).

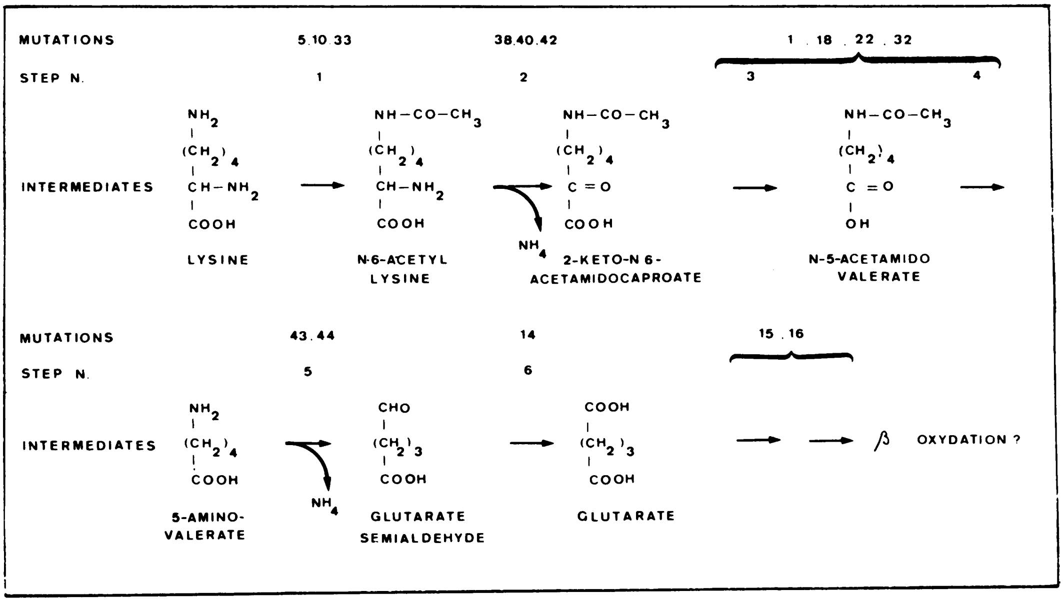

FIG. 4. *Proposed lysine catabolic pathway and the location of mutants.*

We propose that oxidative decarboxylation in the α position (step 3) precedes deamination (step 4) on the main pathway and that 5-aminovaleric acid results. Class 3 mutants are probably affected in these reactions. The fact that mutants of this class belong to three complementation groups could mean that more steps are involved in the transformation of 2-keto-*N*-6-acetamidocaproate into 5-aminovalerate. The inability of these mutants to use lysine as a nitrogen source is in contradiction with their presumptive position in the pathway. This discrepancy has been attributed to regulatory effects, but this hypothesis clearly needs further support. Partial revertants of these mutants, phenotypically lysN$^+$lysC$^-$, are frequently isolated on a lysN$^-$lysC$^-$ background of cells; these might represent strains with defects in the proposed regulatory control and are currently under study.

Degradation of 5-aminovaleric acid probably involves deamination of this compound (step 5, class 4 mutants) and conversion to glutaric acid (step 6). *S. lipolytica* does not normally use glutaric acid as a carbon source, probably because of reduced uptake of this compound by the cells. But after treatment with toluene vapors (1 mn) on a medium containing glutaric acid as a carbon source, W29, class 4 mutants, and one mutant (*lyc14*) of class 5 were able to grow, but poorly, on this medium. *lyc15* and *lyc16* repeatedly failed to grow, and are probably blocked after glutaric acid (the toluene treatment did not prevent these mutants from growing on a glucose medium).

It must be stressed that the proposed scheme may well be an oversimplification. As pointed out above, in most systems studied, the catabolism of both steric forms of lysine occurs via specific pathways. This hypothesis could not be tested here, since *S. lipolytica* does not use the D form of externally added lysine, either as a carbon or nitrogen source or in protein biosynthesis. The occurrence of two separate and isomer-specific pathways, both leading to an assimilable nitrogen or carbon source, is therefore not excluded. But, as monogenic mutants unable to catabolize externally supplied L-lysine can be found, it must be supposed that the existence of the hypothetical "D pathway" is not coupled with an efficient LD-racemase system for lysine (for a different situation, see reference 16).

Regulation of the lysine catabolic pathway was not the aim of this report. The proposed metabolic scheme does not account at all for the inability of the strains to use lysine as a sole carbon and nitrogen source. As 5-aminovaleric acid and, to a lesser extent, *N*-6-acetyllysine can be used in this way, it seems likely that some kind of catabolic regulation is operating on the first step. The fact that class 1 mutants are sensitive to lysine, but only in ammonium-limiting medium, can be taken as an indication of this regulation. Revertants of these mutants, resistant to lysine but unable to catabolize it, have been selected. They will be described elsewhere.

On the other hand, it appears that lysine alone is not the inducer of the pathway insofar as the first steps prior to 5-aminovaleric acid are concerned. We have shown that step 1 is probably induced (or activated) by 5-aminovaleric acid and not by lysine. The following steps are either constitutive or induced by *N*-6-acetyllysine (since class 1 mutants grow well on *N*-6-acetyllysine as a carbon or nitrogen source, when its conversion to lysine can certainly not occur). This hypothesis is further strengthened by the fact that wild-type cells precultivated on glucose and transferred to lysine as a carbon or nitrogen source grow after a long lag period, which is reduced greatly on *N*-6-acetyllysine and even more so on 5-aminovaleric acid.

This lag may represent accumulation of an intermediate product and further induction, rather than metabolic reorientation from glucose to lysine: a lysine-accumulating strain, such as mg-5 (7), grows rapidly on lysine as a carbon source when transferred from a medium containing glucose.

Further work on this aspect of the degradation pathway will also be concerned with the biochemical characterization of some of the presumably concerned activities.

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