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Fatty Acid Desaturase Mutants of *Saccharomyces cerevisiae*

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Genetic and biochemical analyses were conducted on fatty acid mutants of yeast deficient for Δ^9-desaturase activity in the production of palmitoleate and oleate. Two genetic loci were observed and two others are inferred; three of these were represented by respiratory-deficient (petite) strains. All strains were incapable of converting palmitate to palmitoleate and stearate to oleate whether the direct precursor or acetate was followed. All strains were capable of acylating both de novo-produced fatty acids and oleate taken up from the medium into phospholipids and neutral lipids. Two revertants were analyzed which differed in their ability to produce palmitoleate and oleate.

An unsaturated fatty acid (UFA) is required in the cellular composition of all organisms except viruses. Some of these synthesize all their lipid needs; others have specific requirements. In general, microbes and plants occupy the former category and animals the latter. Several microorganisms, however, require a UFA, e.g., a number of bacteria (4, 5, 7, 11, 15, 21, 22), anaerobic yeast (1, 13), mycoplasma (17), and a mutant of *Neurospora* (12). In addition, it is generally agreed that mammals and most insects require certain dietary UFA for normal growth and body maintenance. Silbert and Vagelos (20) isolated and biochemically characterized a fatty acid mutant of *Escherichia coli*. This mutant was not capable of producing palmitoleate (16:1) or *cis*-vaccinate (18:1 Δ^{11}), the naturally occurring UFA found in *E. coli*. They observed that oleate (18:1) could fulfill the UFA requirement. Later (19) their studies were extended to include a number of other fatty acids which could also substitute for the naturally occurring components. Yeast mutants requiring a UFA for growth have also been reported (16). In the present study these mutants were further analyzed genetically and biochemically.

MATERIALS AND METHODS

Genetic analysis. Sporulation of diploids was promoted by first growing the cells overnight on medium containing 5% dextrose, 1% yeast extract, 2.3% Nutrient Agar (Difco), and 0.5% agar, and then transferring the cultures to both types of sporulation media described below. The sporulation culture was incubated for 3 days and then examined for the presence of asci. Spore tetrads were isolated according to the procedure described by Johnston and Mortimer (8). The nutritional requirements of the spore clones were assayed by replica plating onto suitable diagnostic medium.

The following sporulation media were used: (i) 3% potassium acetate, 0.002% raffinose, 2% agar; and (ii) 1% potassium acetate, 0.1% dextrose, 0.25% yeast extract, and 2% agar. The standard liquid culture medium (YEPD) contained (w/v) 1% yeast extract, 2% peptone, and 2% dextrose; 2% agar was added for plate culture. Medium used for scoring the petite phenotype (petite medium) contained 30 g of glycerol per liter, 250 mg of dextrose per liter, 1% yeast extract, 1% peptone, and 2% agar. Minimal medium consisted of 10 g of dextrose per liter, 12.6 g of glycerol per liter, and 6.7 g of yeast nitrogen base per liter, again with 2% agar added for plates. Glycerol and dextrose were obtained from Scientific Products; the other basic culture components were obtained from Difco. Medium termed "synthetic complete" consisted of minimal plus the following (per liter): 16 mg of L-lysine, 16.6 mg of L-arginine, 20 mg of L-methionine, 10 mg of L-histidine, 50 mg of L-leucine, 20 mg of L-tryptophan, 350 mg of L-threonine, 15.8 mg of adenine, and 20 mg of uracil. L-Lysine and adenine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and the remaining additions yielding synthetic complete were from Calbiochem, Los Angeles, Calif. Tween 80, from J. T. Baker Chemical Co., and Triton X-100, from Calbiochem, were used to solubilize fatty acids and were added at 1% (v/v). Fatty acid supplements were provided at 1 g/liter.

Lipid extraction. Cultures for tracer analysis were grown in YEPD-Tween 80 medium with the appropriate tracer-containing component. After 48 hr of growth the cells were collected by centrifugation,

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washed, and freeze-thawed to aid in lipid extraction. Total lipids were extracted by the method of Folch et al. (6) after overnight stirring in 2:1 chloroform-methanol at room temperature. The lipid extract was separated into phospholipid, free fatty acid, and neutral lipid classes as previously described (9). Each of these classes was saponified; the fatty acid fractions were recovered and counted with a Beckman liquid scintillation spectrometer equipped with an external quenching standard. Dual isotope analyses (^3H and ^{14}C) were carried out by the discriminator ratio method (10). Gas-liquid chromatography (GLC) was carried out on fatty acids which were prepared by direct saponification of yeast cells (2 N NaOH) for 1 hr in methanol at reflux temperatures. Methylation was carried out according to the method of Böttcher et at. (3).

Chromatography. GLC was carried out on a Varian Aerograph Autoprep, model 700, equipped with a copper column (4.57 m by 0.63 cm) packed with chromosorb W, 60/80 mesh, coated with 15% diethylene glycol succinate. Samples were washed from collecting tubes into liquid scintillation counting vials. Standard 2,5-diphenyloxazole:toluene was used. Commercially purchased, labeled fatty acids were analyzed by preparative GLC followed by scintillation spectroscopy with the following results: 16:0-*1*-^{14}C, specific activity, 8.0 mc/mmole (Nuclear Chicago), >99% pure; 18:0-*1*-^{14}C, specific activity, 9.19 mc/mmole (Nuclear Chicago), >98% pure; 18:1-*9*, *10*-^3H, specific activity, 3.02 c/mmole (Nuclear Chicago), >99% pure. No tracer-containing impurities were found in acetate-*1*-^{14}C by thin-layer chromatography. Areas of the plate were scraped off, eluted, and counted by scintillation spectroscopy. Standard background for each fatty acid was determined by collecting each component from a fatty acid carrier mixture by preparative GLC followed by scintillation spectroscopy. When 16:0-*1*-^{14}C and 18:0-*1*-^{14}C standards were collected by preparative GLC and then the 16:0 and 18:0 were reinjected and collected again, a measure of 16:0 to 16:1 and 18:0 to 18:1 peak overlap was achieved. Although the GLC recordings of peaks looked clean, there was a range of 0.2 to 1.7% of the counts recovered in the corresponding unsaturated area. Therefore, small amounts of radioactivity appearing in unsaturated positions could usually be accounted for by "bleed-over" from the corresponding saturated fatty acid (Table 1).

RESULTS

Genetic analysis. It is of particular interest to determine the number of segregating genetic loci affecting the Δ^9-desaturation of palmitate and stearate in yeast. Although evidence for four loci are presented, it is not known how many more it may be possible to isolate. The analysis is complicated since three of the four loci are represented by respiratory-deficient strains, and all of these have an additional nutritional requirement. In these studies, respiratory deficiency was defined by inability to utilize nonfermentable substrates, e.g., glycerol, for growth.

Spore viability was greatly increased over that observed previously (16) when the sporulation procedure was followed. Therefore, mutants reported earlier (16) could be subjected to further genetic analysis. All of the mutants tested (KD18, KD20, KD46, KD91, KD115 and KD180) were found to be recessive. In crosses of KD144 to a strain not requiring a UFA for growth (X1687-101B), the UFA requirement was not observed to segregate with any nuclear gene and gave 0:4 segregation (requirement:nonrequirement). Therefore, the UFA requirement in KD144 is tentatively considered to be cytoplasmically inherited.

Regular 2:2 segregation of requirement:nonrequirement was observed in tetrads obtained from a hybrid of KD46 and X1687-101B. In a cross of KD46 to a strain marked by *ol 1*, of seven asci analyzed, three were nonparental ditype and four were tetratype, indicating that the two genes are not linked. Therefore, KD46 bears the newly identified gene *ol 2*. (The diploid formed from KD46 and an *ol 1* mutant was wild type.) Since diploids formed from KD91 or KD180 and strains marked by *ol 2* (from KD46) also required a UFA, the mutations in the strains KD46, KD91, and KD180 are considered allelic and are given the allele designations *ol 2-1*, *2-2*, and *2-3*, respectively.

Regular 2:2 segregation of the lipid requirement was not observed when KD18 was crossed with wild type. Instead, the tetrad classes were of three types, 2:2, 1:3, and 0:4 (nonrequirement: requirement) with a frequency of 1, 13, and 4, respectively. These results would be expected if KD18 were a double mutant; the expected distribution of these tetrad classes for two unlinked genes is 1:4:1. Since the respiratory-deficient phenotype of this mutant was observed to segregate with the UFA requirement, the *ol* genes in this mutant are concluded to be different than *ol 1* (*ol 1* mutants are not respiratory-deficient). Furthermore, in diploids formed from this mutant and an *ol 1* strain, wild-type growth was observed. Wild-type growth was also observed in diploids formed from this mutant and an *ol 2* strain. The tetrad classes among the 14 asci isolated from the cross of KD18 and an *ol 2* strain were of only two types, 1:3 and 0:4 (nonrequirement:requirement), with a frequency of seven each. Therefore, it appears that the *ol* genes in KD18 are neither *ol 1* nor *ol 2* but, instead, represent two additional loci associated with UFA synthesis, *ol 3* and *ol 4*. (The observations are too few for this to be stated as a firm conclusion, and other explanations are possible.)

It was previously reported that the *ol* mutants KD18, KD46, KD91, and KD180 were also

respiratory-deficient. In the present study the respiratory-deficient phenotype segregated with the requirement for a UFA.

Growth characteristics. The mutants can be divided into two groups based on genetic and growth results: group 1 (KD18, KD46, KD91, KD144, and KD180), slow-growing in the presence of a UFA and respiratory-deficient; group 2 (KD20 and KD115), respiratory competent and rapidly growing in the presence of a UFA. In addition, all the group 1 mutants required one of the supplements present in synthetic complete medium but not present in minimal medium. The specific nonlipid nutrient(s) required was not determined. The diploid KD46:KD144 did not require a UFA or a nonlipid supplement for growth, nor was it respiratory-deficient. For group 2 there was no "appreciable" increase in cell number on medium deficient in Tween 80, although residual growth was noted. Approximately one cell division occurred on minimal medium, whereas there was an average of two cell divisions on synthetic complete. Group 2 grew on minimal medium supplemented with highly purified 18:1 Δ^9 *cis* or 18:2 $\Delta^{9,12}$ *cis, cis* but not on 16:0, 18:0, 18:1 Δ^9 *trans* or 18:1 Δ^{11} *cis*. A detailed study of growth rates and replacements by fatty acid structural analogues will be presented elsewhere.

Biochemical studies. Table 1 shows the distribution of ^{14}C from acetate-*1*-^{14}C into fatty acids of the strains analyzed. This reveals the capacity of each strain to synthesize fatty acids de novo and to desaturate these newly synthesized fatty acids. There are several biochemical similarities among the mutants. All of the mutants can elongate acetate to palmitate (16:0); to a limited extent, they subsequently elongate 16:0 to stearate (18:0). In all of the mutants analyzed there is a biochemical block at the desaturation of 16:0 and of 18:0. Furthermore, the chain elongation of 16:0 to 18:0 is reduced to about one-sixth that of the wild type. The distribution of labeled acetate observed for two revertants was different in each case (Table 1). The label distribution for KD20r_1 resembled that of the wild type S288C, whereas KD115r_1 resembled the heterozygous mutant diploid KD46:KD144 to a greater extent. Repeated determinations on these revertants, made over a period of 3 months, gave the same general values. However, it is recognized that fitter revertants may accumulate in such populations over a period of time and that this could lead to changed fatty acid distributions. The genetic and biochemical significances of the observed similarities are at present unknown.

Several radioactively labeled fatty acids were also employed; namely, 16:0-*1*-^{14}C, 18:0-*1*-^{14}C, and 18:1-*9*,*10*-^{3}H. Desaturase mutants grown on these fatty acids yielded fatty acid distributions comparable to those shown in Table 1. When 16:0-*1*-^{14}C was employed, the distribution of ^{14}C

TABLE 1. *Distribution of ^{14}C- from 1-^{14}C-acetate into fatty acids*[a]

Yeast strain	Genotype	Fatty acid (% of total counts/min)					
		<16:0	16:0	16:1	18:0	18:1	16:1/18:1
KD20	*ol 1*	2.3	79.0	0.7	17.8	0.2	
KD115	*ol 1*	4.1	78.4	1.0	16.2	0.3	
KD18	*ol 3; ol 4* (?)	10.0	74.1	0.7	13.4	1.8	
KD144	cytoplasmic (?)	4.1	80.7		15.2		
KD46	*ol 2-1*	5.6	75.5	2.1	13.5	3.3	
KD91	*ol 2-2*	1.6	83.4		15.0		
KD180	*ol 2-3*	2.1	83.1	1.4	12.5	0.8	
KD20r_1[b]	?	2.0	13.7	*8.7*	6.2	*69.4*	0.12
KD115r_1[c]	?	1.7	9.4	*48.6*	2.4	*37.9*	1.3
KD46:KD144	*ol 2-1*/cytoplasmic (?)	1.1	9.4	39.3	2.7	47.5	0.7
S288C		0.4	15.0	11.9	8.3	64.4	0.22

[a] GLC conditions and tracer analysis are described in Materials and Methods. All numbers represent an average of two independent determinations, except as noted; 18:2 and 18:3 are not considered in these analyses since they are minor components in strains derived from the wild-type S288C. All determinations were represented by a total of >5,000 counts/min in the five fractions shown; 16:1/18:1 ratios are valid only where Δ^9-desaturase activity is clearly present. The following strains are respiratory-deficient and also require a nonlipid supplement: KD18, KD144, KD46, KD91, and KD180. The diploid KD46:KD144 does not require a UFA or a nonlipid supplement for growth, nor is it respiratory-deficient.

[b] Average of three determinations.

[c] Average of four determinations.

was almost exactly the same as when acetate-*1*-^{14}C was used; 75 to 85% of the ^{14}C remained as 16:0 and 10 to 15% was elongated to 18:0. When 18:0-*1*-^{14}C was employed, about 90% remained as 18:0 and most of the rest appeared as 16:0, with a small amount in 16:1. Use of 18:1-*9,10*-^{3}H and 18:1-*1*-^{14}C revealed that these mutant strains are capable of further desaturating 18:1 to 18:2 and 18:3, but only slightly. Even in the wild-type strain (S288C), only 3% of the 18:1 was further desaturated to 18:2, with a small amount also going into 18:3. All the mutants derived from S288C (Table 1) were even less efficient in these additional desaturations.

To determine if there were differences between the distribution of long-chain fatty acids synthesized de novo from acetate and a long-chain fatty acid taken up by the organism and subsequently incorporated into lipid classes, two different tracers were employed simultaneously. Table 2 shows the distribution of tracer incorporated into FA from acetate-*1*-^{14}C and 18:1-*9,10*-^{3}H. All of the mutants were capable of acylating both the fatty acids produced de novo and the fatty acids taken up from the medium into ester-containing lipids (Table 2). In general, the distributions of ^{14}C and ^{3}H are about the same, indicating that there is not a great deal of distinction between fatty acids formed de novo and fatty acids incorporated from the medium, as far as incorporation into lipid classes is concerned. Although there are quantitative differences in the distribution of labeled precursors into lipid classes among the strains employed, it is not presently possible to correlate these alterations with structural differences or with meaningful biochemical differences with any degree of confidence.

TABLE 2. *Per cent distribution of fatty acid into lipid classes from 18:1-9,10-*^{3}H and acetate-*1*-^{14}C[a]

Yeast strain	Phospholipid		Free-fatty acid		Neutral lipid	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
S288C	36	49	15	13	49	38
S288C:X1687-101B[b]	62	60	6	6	32	34
KD20	6	10	1	4	93	86
KD115	26	24	16	20	58	56
KD18	10	20	28	26	62	54
KD144	5	14	35	35	60	51
KD46	9	21	21	16	70	63
KD91	11	21	27	25	62	54
KD46:KD46[b]	55	45	25	11	20	44

[a] Acetate-*1*-^{14}C and *18:1-9,10*-^{3}H were employed simultaneously. The lipid classes were purified, and each class was saponified. Fatty acids from these classes were analyzed for tracer content employing the discriminator ratio method for simultaneous ^{3}H and ^{14}C analysis in a liquid scintillation spectrometer (10). All analyses yielded a total of >12,000 counts/min in the three lipid classes analyzed for each tracer.

[b] Diploid: S288C:X1687-101B data is presented for comparison with that of S288C, since both are *ol*$^{+}$ and grow on minimal medium, and with that of the homozygous mutant diploid KD46:KD46.

One interesting correlation revealed by Table 2 is the similarity in fatty acid distribution between the two diploids, S288C:X1687-101B (wild type) and the homozygous mutant KD46:KD46. It may be that yeast diploids, regardless of genotype (KD46:KD46 has the same growth requirements as does KD46), incorporate a greater proportion of their fatty acids into phospholipids than do haploids. The observations of this phenomenon are too few, however, for any firm statement to be made.

DISCUSSION

Extensive biochemistry has been carried out on fatty acid desaturation reactions in the laboratory of Konrad Bloch. In yeast, Bloomfield and Bloch (2) found two fractions capable of carrying out this desaturation: an 8,000 × *g* pellet and a 104,000 × *g* pellet; both showed about the same activity per milligram of protein. The scheme of this reaction (with adenosine triphosphate and Mg^{++} producing reaction 1, and O_2 and reduced nicotinamide adenine dinucleotide phosphate producing reaction 2) was reported as:

(1) 16:0 + Coenzyme A → 16:0 − CoA
(2) 16:0 − CoA → [Oxygen-16:0 − CoA]
(3) [Oxygen-16:0 − CoA] → 16:1 + CoA

This is easily divided into two parts: acylase step (1) and desaturase step (2 and 3). More recently, Nagai and Bloch (14) described an oxygen-dependent, desaturating system in *Euglena*. This system has a minimum of three proteins which are directly involved in desaturase activity.

In the present investigation, all of the mutants employed were capable of acylating both de novo-produced 16:0 and 18:0 and exogenously supplied 18:1 into glycerol esters; therefore, it is doubtful that any are deficient in an acylase enzyme limiting the 18:0 → 18:1 or 16:0 → 16:1 desaturations. In fact, it appears that all the mutants have the same biochemical deficiency or, at least, a deficiency which results in the same biochemical limitations.

Although the number of proteins involved in

the Δ^9-desaturation reaction of yeast is unknown, it seems reasonable, from the work of Bloomfield and Bloch (2) on yeast and Nagai and Bloch (14) on *Euglena*, that there are at least three. In the present investigation, four possible genetic loci were identified, all of which resulted in Δ^9-desaturase deficiency. However, it is also possible that in the group 1 mutants the desaturase deficiency results from their respiratory deficiency, since revertants of these mutants show a concomitant loss of the respiratory-deficient and UFA-deficient phenotypes. A component of mitochondria might be required for UFA synthesis as well as normal mitochondrial function. Thus, a cell deficient in this component would be respiratory-deficient and UFA-supplementable. The *ly 6* and *ly 8* mutants investigated by Sherman (18) are similar in that they are lysine auxotrophs and respiratory-deficient. These results indicate that the mitochondria are important not only in cellular respiration but also in the metabolism of various molecules. The pleiotropic effect of the *ol 2*, *3*, and *4* mutants further corroborate this idea since they also require one of the amino acids or bases in synthetic complete medium.

Although no other respiratory-deficient mutants have been reported to require a UFA for growth, this may have been due to the methods of isolation; respiratory-deficient mutants that are ol^+ may still retain the component required for UFA synthesis. However, the *ol 1* mutants KD20 and KD115 are not respiratory-deficient nor do they require any amino acids or bases; thus, they appear to be true desaturation mutants.

The 16:1/18:1 ratio varies widely throughout the large number of organisms which produce both these fatty acids. For example, in another fungus, *Neurospora*, the 16:1/18:1 ratio is about 0.1; in *Drosophila* the ratio is close to 1.0, and in most higher animals the ratio is around 0.2. In yeast both the genetic composition and the growth medium alter this ratio. If culture conditions are kept constant, however, the ratio remains about the same in independent determinations for any given strain, as might be expected. Both KD20 and KD115 were derived from the same haploid wild strain, S288C, which was growing under identical culture conditions when each was isolated. The ratios for a revertant of each were different. The spontaneously occurring revertant of KD20, KD20r_1, yielded a 16:1/18:1 ratio of 0.12, whereas a comparable revertant of KD115, KD115r_1, gave a ratio of 1.3; these two ratios differ by a factor of 10. Since all seven of the mutants reported were blocked at the desaturations of both 16:0 and 18:0 and both revertants studied recovered both of these steps simultaneously, it seems clear that both reactions are mediated by the same enzyme. The work of Bloomfield and Bloch (2) on the yeast Δ^9-desaturase enzyme also indicates that both activities are located together. Since Δ^9-desaturase activity was restored to 16:0 and 18:0 differentially in the two revertants, it is likely that the corrected enzymes were not identical and thus had different specificities for the two substrates.

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