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Unsaturated Fatty Acid Mutants of *Neurospora crassa*

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Unsaturated fatty acid (*ufa*) auxotrophs of *Neurospora crassa* were obtained by treatment of conidia with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by isolation on media containing polyunsaturated fatty acids suspended in Tergitol NP-40. The 24 mutants for which reisolates were obtained from crosses with wild type were assigned to two complementation classes, *ufa-1* and *ufa-2*, located on linkage group V. Unsaturated fatty acids with varying degrees of unsaturation, chain length, and double-bond position as well as different steric configurations were tested for growth requirements.

Genetic analysis as a tool for dissecting the requirements and the role of fatty acyl groups in cellular membranes has been initiated in several systems, notably *Escherichia coli* and yeast (6, 15). Mutations affecting the synthesis (3, 4, 14) and desaturation (12, 17) of fatty acids have been reported in both organisms. Although *E. coli* and yeast can utilize exogenous polyunsaturated fatty acids (C_{18}-C_{22}) under the appropriate conditions (6, 16), both organisms contain a single desaturase and synthesize only monounsaturated fatty acids (15). Extension of these genetic analyses to organisms that normally contain polyunsaturated fatty acids should greatly enhance our understanding of more complex systems of fatty acid desaturation in addition to the influence of fatty acid composition on membrane structure and function. With this in mind, we undertook a search for unsaturated fatty acid (*ufa*) mutants in *Neurospora*. This communication is concerned with the isolation of *ufa* mutants of *Neurospora* and a description of their genetic and physiological characteristics.

MATERIALS AND METHODS

Chemicals. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Aldrich Chemical Co., Milwaukee, Wis. Tergitol NP-40 and Tween 80 were obtained from Sigma Chemical Co., St. Louis, Mo. Fatty acids were the products of Analabs, Inc., North Haven, Conn.

Cultures and crosses. The wild-type RL3-8A was obtained from The Rockefeller University stock collection; the inositol mutant, *inl* (FGSC 498), and *alcoy* (FGSC 997 and 998) were supplied by the Fungal Genetics Stock Center, Humbolt State College, Arcata, California. Crosses were carried out by standard techniques (2) on crossing medium (19) at 25°C. Media were supplemented with 0.1% Tween 80 when the *ufa* mutants were employed as protoperithecial parents.

Stock cultures were maintained at 4°C on agar slants of minimal medium (18) plus supplements where appropriate. Stock cultures of the *ufa* mutants were supplemented with 0.1% Tween 80. The long-term viability of these strains was considerably greater on Tween 80-containing medium than on medium containing fatty acid-detergent complexes (see below). The *ufa* mutants were grown at 34°C on pure fatty acid supplements in media containing 1% of the nonionic detergent, Tergitol NP-40. Pure fatty acids were suspended in the detergent at 60°C by gentle swirling. After dissolving in a minimal volume of medium, the fatty acid-detergent mixture was added to sterile media of syringes equipped with Swinnex filters (0.45 μm). Growth rates were measured in "race" tubes (13).

Mutagenic treatment. Conidia of *inl* were treated with MNNG essentially as described by Malling and DeSerres (9). In brief, conidia were suspended in 0.1 M sodium-potassium phosphate buffer, pH 6.0, at a concentration of 2×10^7 conidia/ml. Freshly prepared MNNG was added to a final concentration of 100 μM, and the suspension was agitated on a reciprocal shaker (102 strokes/min) at 25°C for 1 h in the dark. The reaction mixture was diluted fivefold with distilled water and washed three times in the same.

Isolation of *ufa* mutants. The *ufa* auxotrophs were isolated by the technique of filtration enrichment (1). MNNG-treated conidia were suspended in distilled water at 2×10^7 spores/ml. These were inoculated into flasks (250 ml) containing 100 ml of minimal medium (18), 1.5% sorbose, 0.2% glucose, and 5 mg of inositol per liter at a final concentration of 2×10^5 conidia/ml. The flasks were agitated on a reciprocal shaker (102 strokes/min) at 25°C. After 12 to 14 h of incubation, the contents of the flasks were filtered through glass wool to remove wild-type growth and then filtered periodically as visible growth occurred. Growth usually ceased after 72 h. Incubation was continued for an additional 24 h, after which time the cultures were again filtered. Conidia were collected by centrifugation and suspended in a small volume of distilled water. Portions of the final conidial suspension were plated on agar

medium of composition similar to that employed in the above selection step, but supplemented with 1% Tergitol NP-40 plus 1 mM unsaturated fatty acid(s). Colonies obtained after incubation at 34°C were transferred to slants containing inositol (50 mg/liter) and 0.1% Tween 80. Initial screening for *ufa* auxotrophs was carried out by examining the growth requirement of each isolate for Tween 80. Those isolates unable to grow in the absence of Tween 80 were retained and subsequently tested for their growth specificities on media containing 1 mM fatty acids suspended in 1% Tergitol NP-40.

Complementation tests. Heterokaryons were generated between *ufa* mutants by pairwise inoculation onto solid minimal medium supplemented with 50 mg of inositol per liter. The complementation tests were performed with the original *ufa* isolates prior to backcrossing with wild type. This procedure was followed in order to avoid introduction of heterogeneity at the heterokaryon-incompatibility loci from the wild-type background.

RESULTS

Mutant selection. Wild-type *Neurospora* synthesizes significant amounts of C_{16} and C_{18} unsaturated fatty acids when grown on minimal medium (6). Mutants were selected on agar medium containing these fatty acids. Table 1 shows the fatty acid composition of the different selection media and the number of mutants recovered on each. A total of 43 *ufa* auxotrophs were collected. Of these, five were subsequently discarded because of poor growth. This report is concerned with 24 of the remaining 38 original isolates for which *ufa* progeny have been obtained from backcrosses to wild type.

The recovery of *ufa* mutants is dependent upon both the mutagen and isolation technique employed. Substitution of ultraviolet (>90% killing) or nitrous acid (>50% killing) for MNNG as the mutagen substantially reduced the number of *ufa* mutants obtained. In addition, all of the ultraviolet- and nitrous acid-induced auxotrophs were leaky and readily reverted. The effectiveness of the filtration enrichment step was also evident. Attempts to isolate *ufa* mutants by the inositolless death technique (8) from MNNG-treated conidia failed. Of these various procedures, MNNG mutagenesis followed by filtration enrichment proved to be the only effective combination for producing high yields of *ufa* mutants. With the procedure outlined (see above), the proportion of *ufa* mutants approached 25% of the germinated colonies on the selection media. A high degree of selectivity for *ufa* auxotrophs was apparent in that only two saturated fatty acid-requiring strains were detected among the colonies exhibiting a Tween requirement for growth. (Tween 80 is an excellent supplement (5) for the *cel* mutant, a saturated fatty acid-requiring strain).

Complementation and linkage. The complementation map for the *ufa* mutants is shown in Fig. 1. As indicated, two complementation classes were detected. The *ufa-2* locus can be represented as a linear array of seven complementation groups, provided that group VII is drawn as two separate defective regions. The *ufa-1* locus consists of a single mutant. This strain was the only representative for this locus out of the original 43 *ufa* isolates recovered.

Linkage of *ufa* was assessed from crosses to the *alcoy* strain (11). Analysis of random spores from *ufa inl* × *alcoy* indicated linkage of *ufa* to *cot-1*. Location of *ufa* on linkage group V was

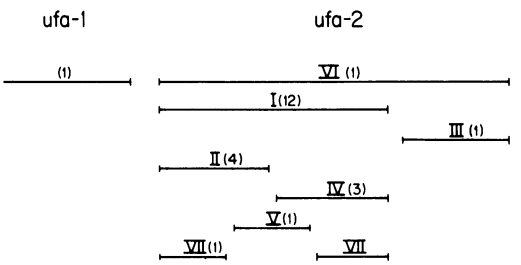

FIG. 1. *Complementation map for ufa mutants in N. crassa. The number of mutants representing each complementation group is given in parenthesis.*

TABLE 1. *Number of ufa mutants obtained on various selection media*[a]

Expt	Fatty acid in selection medium	No. of mutants	No. of colonies examined	(%) *ufa* mutants
1	16:1Δ^9 *cis*	5	101	5.0
2	18:1Δ^9 *cis*	6	119	5.0
3	18:2$\Delta^{9,12}$ *cis, cis*	4	118	3.4
4	18:3$\Delta^{9,12,15}$ *cis, cis, cis*	3	20	15.0
5	18:2$\Delta^{9,12}$ *cis, cis* + 18:3$\Delta^{9,12,15}$ *cis, cis, cis*	8	31	25.8
6	18:1Δ^9 *cis* + 18:2$\Delta^{9,12}$ *cis, cis* + 18:3$\Delta^{9,12,15}$ *cis, cis, cis*	17	78	21.8
Total		43	467	

[a] The concentration of each unsaturated fatty acid was 1 mM.

determined from the linkage relationship between *ufa* and *inl*. In all crosses to wild type or *alcoy*, the *ufa* phenotype was transmitted with a low frequency, which ranged between 3 and 21%. The linkage group V markers (*cot*$^+$ and *inl*) contributed by the *ufa* parent in crosses to *alcoy* were also recovered in low yields (Table 2), whereas markers for other linkage groups (*al*, *ylo*, and mating type) segregated in a 1:1 manner. Selective reduction of the former markers in addition to *ufa* is consistent with the location of *ufa* on linkage group V. The *ufa-1* mutant and mutants representing complementation groups that span the entire *ufa-2* region were found to exhibit similar linkage relationships.

Growth requirements of *ufa* mutants. As expected, saturated fatty acids ($C_{12}-C_{18}$) supported no growth. Of the unsaturated fatty acids synthesized by wild-type *Neurospora*, optimal growth was obtained with 18:1Δ^9 *cis* as the sole fatty acid supplement. The efficiencies of 18:2$\Delta^{9,12}$ *cis, cis*, and 16:1Δ^9 *cis* were similar to 18:1Δ^9 *cis*; however, 18:3$\Delta^{9,12,15}$ *cis, cis, cis* consistently restored less growth than the mono- or diolefinic acids. Figure 2 illustrates typical growth curves for wild type and two *ufa* mutants as a function of 18:1Δ^9 *cis* concentration. All cultures contained 1% Tergitol NP-40. This level of detergent allowed the growth of wild type, but at 25% of the rate of cultures without detergent. Supplementation of wild-type cultures with 18:1Δ^9 *cis* produced no detectable changes in growth rate. In contrast, growth of the *ufa* mutants was found to be a linear function of 18:1Δ^9 *cis* concentration up to 0.75 mM. Optimal growth rates, obtained at 18:1Δ^9 *cis* levels ranging between 1 and 2 mM, were comparable to that of wild type grown in detergent-containing media.

The growth responses of the *ufa* mutants to different fatty acid supplements are summarized in Table 3. Mutants from all complemen-

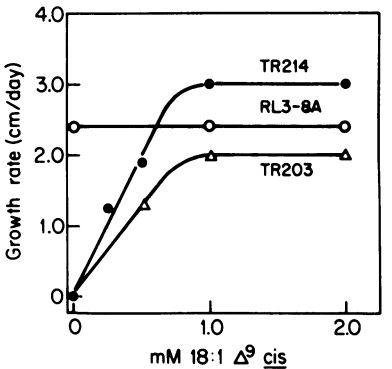

FIG. 2. *Growth rates of ufa mutants and wild type as a function of 18:1Δ^9 cis concentration. TR214 and TR203 are ufa-2 mutants from complementation groups III and II, respectively. RL3-8A is the wild-type strain.*

TABLE 2. *Recovery of markers from a cross of ufa to alcoy*[a]

Progeny	No.	(%) Recovered	(%) Expected[b]
ufa	11	8.0	50
cot	103	75.2	50
inl	31	22.6	50
al	72	52.6	50
al$^+$[c]	30	21.9	25
ylo	35	25.5	25
a	69	50.4	50
Total[d]	137		

[a] Zygote genotype: *ufa-2; inl, a* × *alcoy*[T(I, II) *al-1*; T(IV, V) *cot-1*; T(III, VI) *ylo-1*; A]. The symbols *a* and *A* represent complementary mating-type alleles; *inl* represents the requirement for inositol, and *al*, *ylo*, and *cot* denote the albino, yellow, and temperature-sensitive colonial markers, respectively. T indicates a translocation.

[b] A 2:1:1 segregation of *al*, al$^+$, and *ylo* is expected because of the dominance of *al* to *ylo*.

[c] The symbol al$^+$ denotes the wild-type phenotype.

[d] Germination rate was 82%.

TABLE 3. *Fatty acid growth response*[a]

Fatty acid supplement	Mutants response[b]		
	ufa	Yeast[c] *ole-1*	*E. coli*[d] *fab-A*
12:0	0	0	–
14:0	0	0	–
16:0	0	0	–
18:0	0	0	–
14:1Δ^9 *cis*	0	+	+
16:1Δ^9 *cis*	+	+	+
16:1Δ^9 *trans*	+	+	+
18:1Δ^6 *cis*	0	+	+
18:1Δ^9 *cis*	+	+	+
18:1Δ^9 *trans*	0	0	+
18:1Δ^{11} *cis*	+	+	+
18:2$\Delta^{9,12}$ *cis, cis*	+	+	+
18:2$\Delta^{9,12}$ *trans, trans*	0	+	–
18:3$\Delta^{6,9,12}$ *cis, cis, cis*	+	+	–
18:3$\Delta^{9,12,15}$ *cis, cis, cis*	+	+	–
20:1Δ^{11} *cis*	0	+	+
20:4$\Delta^{5,8,11,14}$ *cis, cis, cis, cis*	+	+	–

[a] The *ufa* isolates were inoculated onto slants of minimal agar medium containing 50 mg of inositol per liter, 1% Tergitol NP-40, and 1 mM of the appropriate fatty acid. Growth was scored after 3 to 4 days at 34°C.

[b] Symbols: 0, No response; +, response; –, no data available.

[c] Data from Wisnieski and Kiyomoto (20).

[d] Data from Silbert et al. (16).

tation groups had similar growth requirements. For comparison, similar data for the yeast and *E. coli* unsaturated fatty acid mutants are also included in Table 3. There is good agreement between the *ufa* mutants of *Neurospora* and the yeast and *E. coli* mutants in terms of fatty acid specificities. Three notable exceptions are evident. Unlike the yeast and *E. coli* mutants, the growth requirements of the *ufa* mutants are not satisfied by 14:1Δ^9 *cis*, 18:1Δ^6 *cis*, or 20:1Δ^{11} *cis*. The *Neurospora* strains are further distinguished from the yeast mutant by their inability to utilize 18:2$\Delta^{9,12}$ *trans*, *trans* and from the *E. coli* mutant by lack of growth when supplemented with 18:1Δ^9 *trans*.

DISCUSSION

Lein and Lein (7) first reported an 18:1Δ^9 *cis*-requiring strain of *Neurospora*, which was subsequently lost. Another auxotroph of *Neurospora* that grew when supplemented with Tween 80 was described by Perkins et al. (10), but it was shown to be a chain elongation mutant with a saturated fatty acid requirement for growth (5). Reported here for the first time is a highly selective and efficient procedure for isolating unsaturated fatty acid (*ufa*) mutants in *Neurospora*. Supplementation experiments with pure fatty acids clearly demonstrate that the *ufa* strains have an absolute requirement for unsaturated fatty acids. The structural features of a growth supporting unsaturated fatty acid appear to be more stringent in *Neurospora* than in yeast or *E. coli*. Minimally, these include: (i) a double bond in the *cis*-configuration at either the Δ^9 or Δ^{11} position and a chain length of 16 or 18 carbons; (ii) a double bond in the *trans* configuration at the Δ^9 position and a chain length of 16 carbons; or (iii) multiple *cis* double bonds interrupted by methylene bridges. The requirements of the *ufa* mutants, therefore, are distinct from the *cel* mutant (5). These results suggest the possibility of isolating mutants with blocks in the different steps of unsaturated fatty acid synthesis in eukaryotes containing complex mixtures of mono- and polyunsaturated fatty acids. That the *ufa* mutants can be localized to two complementation classes is of interest. Considering the complexity of the pathway for fatty acid desaturation (Fig. 3) and the number of other reactions contributing to the synthesis of unsaturated fatty acids, mutations affecting any number of genes would be expected to result in a *ufa* phenotype. Clearly, isolation of additional *ufa* mutants in *Neurospora* and further genetic studies are warranted to determine the number of different gene products that participate in the synthesis of unsaturated fatty acids.

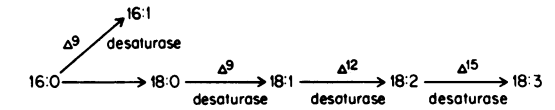

FIG. 3. *Presumed pathway for the biosynthesis of unsaturated fatty acids in N. crassa. Abbreviations: 16:1, 16:1Δ^9 cis; 18:1, 18:1Δ^9 cis; 18:2, 18:2$\Delta^{9,12}$ cis, cis; 18:3, 18:3$\Delta^{9,12,15}$ cis, cis, cis.*

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