ISOLATION AND CHARACTERIZATION OF TEMPERATURE-SENSITIVE RESPIRATORY MUTANTS OF *NEUROSPORA CRASSA**

THAD H. PITTENGER AND DAVID J. WEST

Division of Biology, Kansas State University, Manhattan, Kansas 66506

Manuscript received October 27, 1978
Revised copy received June 8, 1979

ABSTRACT

Filtration-enrichment and inositol-less death methods of mutant isolation, coupled with a screen for cyanide-insensitive respiration, proved to be highly efficient methods for isolating temperature-sensitive (ts) nuclear Neurospora mutants having defective respiration. Eighteen different ts respiratory mutants have been isolated. Most of them are pleiotropic and defective in one or more of the following phenotypes: cytochrome aa_3, *b*, and *c* (individual or multiple defects); oligomycin inhibition of ATPase activity; respiration and its inhibition by KCN and salicyl hydroxamic acid; and growth rates in liquid and solid media at 25° and 38°. Among these mutants are the first cytochrome *c* mutant of Neurospora and an extranuclear ts ATPase mutant. An added bonus was the fact that over half of the mutants were affected either in ribosome assembly or in protein synthesis in the mitochondrion. We have yet to find any mutants completely lacking activities associated with the respiratory chain. However, the wide spectrum of mutants isolated here, along with those currently available, constitutes a considerable resource for investigating respiration in obligate aerobes.

BOTH yeast and Neurospora have figured prominently in studies on mitochondriogenesis. Early efforts to understand this process emphasized genetic analysis of mitochondrial function. Progress was limited, due in part to excessive preoccupation with extranuclear mutants displaying a rather narrow range of phenotypes. In the meantime, biochemical analyses employing selective inhibition of mitochondrial or cytoplasmic protein synthesis indicated that only a small fraction of mitochondrial protein was actually synthesized within the mitochondrian and, therefore, presumably coded for by mitochondrial DNA (for review, see SCHATZ and MASON 1974). Consequently, genetic analysis continued to be a promising avenue of approach to questions of mitochondrial structure and function, but a more diverse group of mutants, both nuclear and extranuclear, was needed for these studies.

TZAGOLOFF, AKAI and NEEDLEMAN (1975); TZAGOLOFF *et al.* (1975) were among the first to enjoy considerable success with yeast in selecting both nuclear and extranuclear mutants having a variety of deficiencies in mitochondrial struc-

* Contribution No. 79-59-j, Division of Biology, Agricultural Experiment Station, Kansas State University, Manhattan, Kansas.

ture and function, but retaining the capacity for mitochondrial protein synthesis. Selection of these mutants, designated *mit*$^-$, was much facilitated by the ability of yeast to use fermentation as the sole mode of energy metabolism.

Neurospora, on the other hand, presented a difficulty not encountered with yeast. It is an obligate aerobe and, although nuclear and extranuclear mutants could be isolated (BERTRAND and PITTENGER 1969, 1972; BERTRAND *et al.* 1977), they tended to be leaky, and there were few genetically distinguishable classes. Ostensibly, many potentially interesting mutants would be lethal if they were deficient in some component of the indispensible respiratory chain. Consequently, we considered that the selection of conditional lethal mutants might provide suitable material for conducting a genetic analysis of respiratory function in this organism.

In this paper, we describe the isolation and preliminary genetic and biochemical characterization of a group of temperature-sensitive (ts) respiratory mutants of *Neurospora crassa*. The respiratory defective mutants among the ts isolates were identified by screening the isolates for cyanide-insensitive respiration—a property characteristic of an alternate oxidase system induced by a variety of deficiencies in the cytochrome-mediated respiratory chain (LAMBOWITZ and SLAYMAN 1971; LAMBOWITZ, SMITH and SLAYMAN 1972b; EDWARDS and KWIECINSKI 1973; EDWARDS, ROSENBERG and MARONEY 1974). In addition to isolating mutants exhibiting a wide range of deficiencies associated with the respiratory chain, we recovered a significant number of mutants that are deficient in mitochondrial ribosome assembly (or in other functions affecting mitochondrial protein synthesis) and that, consequently, have modifications in one or more of the respiratory complexes of the inner mitochondrial membrane. A report on one nuclear mutant having a ts defect in cytochrome *b* and the activity of $CoQH_2$-cytochrome *c* reductase has already been published (WEST and PITTENGER 1977). Recently NARGANG, BERTRAND and WERNER (1978) have isolated cytochrome aa_3 mutants in *Neurospora crassa*, some of which are temperature sensitive.

MATERIALS AND METHODS

Strains and media: The wild type of strains 74-OR23-1A and 74-OR8-1a and mutant strains *inl* [83201(t)] and inl; *ylo-1* [83201(t); Y30539y], were obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, California 95221. Liquid Vogel's minimal medium (VOGEL 1964) with 2% sucrose (hereafter referred to simply as Vogel's medium) was used for the growth of mycelia from which mitochondria were to be isolated, and the same medium with 2% agar was used in growth tubes. In studies involving whole-cell respiration, 2% glucose, autoclaved separately from the salts and later combined, was used instead of sucrose. A medium containing Fries salts (BEADLE and TATUM 1945) with 2% glucose, 2% agar and 0.4% Difco Proteose Peptone #3 (FGPP medium) was used to maintain slant cultures; this medium also was used in 50-ml lots in 250-ml Erlenmeyer flasks to grow conidia used in whole-cell respiration and as inocula for the large-volume mycelial cultures. Sexual crosses were made on the medium of WESTERGAARD and MITCHELL (1947).

Selection of mutants by filtration enrichment: The basic procedure was that of WOODWARD, DEZEEUW and SRB (1954). Ten-day-old conidia of the wild-type stain 74-OR8-1a were irradiated with a germicidal lamp to 42% survival. The conidia were then incubated in Vogel's medium

with rotary shaking for 27 hr at 40°. During this period, the conidial suspension was filtered at six different times through four layers of cheesecloth to remove germinated conidia, then finally concentrated on a millipore filter, resuspended and plated on minimal sucrose-sorbose medium. Following incubation at 27°, 469 colonies were isolated from the plates to slants of Vogel's medium. We then tested 400 of these isolates, which showed good conidia formation at 27°, for growth at 40° in liquid Vogel's medium, and 41 of the isolates failed to grow. Those that failed to grow were subsequently used to inoculate 500-mm long growth tubes. After two days at 27°, the tubes were shifted to 40°. Isolates showing a significant reduction in a growth rate over the next several days were saved for further study. This procedure yielded 24 mutants that had marked ts growth.

Selection of mutants by ionsitol-less death: Inositol-requiring mutants of Neurospora start to grow on minimal medium and then die. Isolation of mutants by the inositol-less death technique is based on the observation that double mutants containing an inositol allele plus an induced second auxotrophic mutation survive longer on minimal medium than does the inositol-requiring mutant by itself (LESTER and GROSS 1959). To select ts mutants by this technique, we used a strain requiring inositol only at elevated growth temperatures, *inl-t* [83201(t)]. We made an assumption, subsequently supported by the mutants isolated in this experiment, that ts respiratory mutants could be selected by the inositol-less death procedure just as effectively as auxotrophic mutants.

Conidia of the inositol-requiring mutant, irradiated with UV to 40% survival, were plated on Vogel's sucrose-sorbose medium. Plates were incubated at 35° for 40 hr, then shifted to 25° for three additional days of growth. Of 600 colonies isolated to minimal slants at 25°, we selected 313 that grew well and formed normal amounts of conidia within a week to test further for growth on inositol-supplemented liquid medium at 40°. Of the isolates, 114 showed little or no growth at that temperature. These presumptive ts mutants were subsequently tested on inositol supplemented medium in growth tubes incubated first at 25°, and then transferred to 38°. Of the isolates, 57 showed a significant reduction in growth rate after the shift to 38° and were saved for further study.

Respiration measurements: To measure whole-cell respiration, we inoculated two 125-ml Erlenmeyer flasks containing 40 ml of Vogel's medium plus 2% glucose with conidia to a final density of 5×10^6 per ml. Inoculated flasks were first held overnight at 8° and were then maintained at 27° for three hr in a shaking water bath to enhance germination and early growth at this permissive temperature. One of the duplicate flasks was then shifted to a 38° bath, and respiration was monitored in both cultures throughout the day. That was done primarily to determine whether induction of the alternate oxidase was temperature sensitive. (Only those respiration rates from cultures that had been growing nine to ten hours are reported in this paper.) Respiration was measured by first taking 10 ml samples from the shake cultures, bringing them to 38° in a water bath, and simultaneously bubbling air through them for several minutes. Aliquots of 1.5 ml were then placed in the reaction vessel of a Gilson Oxygraph equipped with a Clark oxygen electrode, agitated with a magnetic stirring bar, and maintained at 38°. The rate of oxygen utilization was measured, and the specific rate of respiration was calculated and expressed as μl 0_2 per hr per mg dry weight. Assays were done in triplicate. Inhibition of cytochrome-mediated respiration was measured by adding 20 μl of a 1 mg per ml solution of KCN (prepared fresh daily) in distilled water. Respiration caused by the alternate oxidase pathway not mediated by the cytochromes (LAMBOWITZ and SLAYMAN 1971) was assessed by inhibiting it with 10 μl of a 1 mg/ml solution of salicyl hydroxamic acid (SHAM) in absolute ethanol. In many cases the conidial samples had to be diluted prior to aeration and assay. Cell suspensions used in the replicate assays of each culture were pooled and harvested by vacuum filtration on preweighed millipore filters. The samples were air dried overnight at 38° and weighed to obtain the dry weights of the cells.

Growing mycelium: Mycelium used as a source of mitochondria was grown in 2800-ml Fernbach flasks, each containing 1000 ml of Vogel's medium, on a rotary shaker at 190 rpm. Flasks were inoculated to a final density of 10^6 per ml with seven- to ten-day-old filtered conidia grown on FGPP medium. Growth times varied because of the different growth rates charac-

teristic of the wild-type and mutant strains at different temperatures. An effort was made to harvest cultures in the exponential phase of growth when most cultures were considerably less than 24 hr old. In a typical experiment, one flask would be grown continuously at 25°, while a second would be grown for seven to eight hours at 25° and then shifted to 38° for a similar period of growth.

Preparing mitochondria: Mitochondria were prepared by the snail-gut enzyme procedure of GREENAWALT, HALL and WALLIS (1967), as modified by LAMBOWITZ, SMITH and SLAYMAN (1972a).

Enzyme assays: Cytochrome oxidase (EC 1.9.3.1) and $CoQH_2$-cytochrome *c* reductase were assayed as described by WEST and PITTENGER (1977), except that aliquots of frozen-thawed mitochondria were not preincubated.

ATPase (EC 3.6.1.4) activity was routinely measured by a procedure in which ATP hydrolysis was enzymatically coupled to the oxidation of NADH. The assay mixture contained 0.05 M Tris-HCl, (pH 8.25), 10 mM KCl, 3 mM ATP, 3 mM phosphoenolpyruvate, 3 mM $MgCl_2$, 0.23 mM NADH, 5 μg antimycin A in 5 μl methanol, 5 μl pyruvate kinase (Sigma Type 1), and 5 μl lactate dehydrogenase (Sigma Type II) in a final volume of 3.0 ml. Reaction was initiated by adding a frozen-thawed suspension of mitochondria. Assay temperature was 28°. Reaction rate, determined from the decrement in absorbence at 340 nm, was corrected for NADH oxidized in the absence of mitochondria. To assess the oligomycin sensitivity of the ATPase, we added 10 μg of oligomycin in 5 μl methanol. In some cases, ATPase was also assayed by directly measuring the P_i released during ATP hydrolysis; in such cases NADH, antimycin A and lactate dehydrogenase were omitted. The reaction was allowed to proceed for ten min at 28° and was then terminated by adding TCA. The mixture was centrifuged, and the P_i content of the supernatant was determined colorimetrically using Sigma Kit #670. Controls lacking ATP and mitochondria were run. Results obtained with the two assay systems were in good agreement.

Cytochrome spectra: Difference spectra of reduced, as opposed to oxidized, suspensions of mitochondria were determined as previously described (WEST and PITTENGER 1977).

RESULTS

Cyanide resistance in mutants: ts mutants isolated by the filtration enrichment and inositol-less death techniques were screened for cyanide-resistant respiration, which is mediated by an alternate oxidase known to be induced when the cytochrome chain is defective (LAMBOWITZ and SLAYMAN 1971; LAMBOWITZ, SMITH and SLAYMAN 1972b; EDWARDS and KWIECINSKI 1973; EDWARDS, ROSENBERG and MARONEY 1974).

Of the 24 ts mutants obtained with the filtration enrichment procedure, five were found to have cyanide-insensitive respiration and were thus judged to be deficient in cytochrome mediated respiration. Each of the mutants was crossed as the female parent to the wild-type strain 74–OR23–1A. Progeny from the crosses were tested for growth at 25° and 38°. For four of the mutants (A8, A10, A13 and A84), the progeny segregated approximately 1:1 for ts and non-ts growth, thus permitting their designation as single gene nuclear mutants.

The fifth mutant (C93) was also crossed as the female parent to the wild type, but there was no apparent segregation for differences in growth rates among the 40 progeny tested. There were, however, significant differences in growth of all isolates at 25° as compared to 40°; all grew rapidly at 25° and slowly at 40°. We selected 15 of the isolates at random and measured their respiration in the presence and absence of cyanide; all showed cyanide-resistant respiration only

when grown at 40°. One of the isolates was then crossed as the male parent to the wild-type strain; again, there was no segregation among the progeny, but 32 isolates from this cross all grew rapidly at both 25° and 40°. We selected 15 of these isolates at random and measured their respiration after growth at 40°; in every case, cyanide inhibited respiration. This maternal inheritance and lack of segregation for ts growth and cyanide-insensitive respiration permitted the identification of C93 as an extranuclear mutant.

The 57 confirmed ts isolates obtained by the inositol-less death procedure were tested for cyanide-insensitive respiration and 12 of them proved to have this characteristic. In a separate experiment involving the mutant *inl*; *ylo-1* [83201(t); Y30539y] an additional mutant, RK3–21, was isolated by the same technique and was included in the final group of mutants.

To study the genetic basis of induced ts respiratory-deficient mutations in strains already having a ts requirement for inositol (*inl-t*), we first had to separate the two mutations. We did that by crossing the presumed double mutants as the female parent to wild type, isolating random ascospores from the cross, and then identifying ts isolates without an inositol requirement from among the segregating progeny. The scheme below indicates the growth behavior most generally observed among the segregating progeny:

Genotype		Growth at 38°	
		— Inositol	+ Inositol
+	+	+	+
+	*inl-t*	—	+
respiratory *ts*	—	trace	trace
respiratory *ts*	*inl-t*	—	trace

Though not all ts respiratory defective mutants were easily distinguished from the double mutants by this simple growth test, the scheme did facilitate preliminary classification of the progeny. In particular, progeny with 25% wild-type segregants at 38° helped us identify quickly those crosses with two genes segregating. In some cases respiration studies, including tests for cyanide-sensitivity, plus long-term growth experiments at 38° in the presence and absence of inositol, were used to distinguish genotypes. Ultimately, it was a further cross of the presumed individual ts mutant with wild type, and a 1:1 segregation among the progeny, that confirmed that ts growth and cyanide-resistant respiration were indeed inherited together as a consequence of mutation in a single nuclear gene. In one case, 289–63, in which infertility and poor growth earlier had made it difficult for us to analyze segregating progeny properly, we found that ts growth and respiratory defects were caused by separate nuclear mutations.

Growth studies: Mutants reisolated after being crossed to the wild type strain were tested for ts growth in two ways. One method involved the use of growth tubes; the other involved determination of mycelial dry weights following growth in liquid medium. When growth tubes were used, they were inoculated with conidia and incubated at 28°. The position of the growing front was marked daily. After three days at 28°, the incubation temperature was raised to 38° and daily growth continued to be recorded.

TABLE 1

Growth of mutant isolates in growth tubes and liquid culture at 25° and 38°

Strain*	Growth-tube data: mm growth last day @ 28°†	Growth on successive days @ 38° (expressed as fractions of 28° growth): 1	2	3	Dry weights (mg) of 24-hr mycelial cultures‡: 25°	38°	Ratio (38°/25°)
740R23-1A	95	0.89	0.94	—	363	473	1.30
740R8-1a	104	0.66	0.63	0.53	313	358	1.14
289-56 (324-7A)	103	0.10	0	0	248	76	0.30
A84 (303-8A)	100	0.10	0	0	314	11	0.036
293-18 (326-13A)	95	0.26	0	0	104	73	0.70
289-67 (345-2a)	80	0.59	0	0	225	38	0.17
A13 (302-5a)	50	0.56	0	0	318	143	0.45
A10 (301-8A)	79	0.52	0	0	135	138	1.02
289-4 (322-11a)	83	0.35	0.07	0	96	114	1.18
299-9 (343-38)	96	0.47	0.06	0	314	70	0.22
289-52 (315-6a)	86	0.58	0.02	0	330	36	0.11
291-30 (335-3A)	103	0.50	0.12	0	111	13	0.12
A8 (300-2A)	100	0.54	0.19	0	318	65	0.20
297-24 (340-9a)	91	0.54	0.24	0	160	103	0.64
291-50 (339-2a)	75	0.52	0.31	0	74	16	0.22
RK3-21 (360-1a)	95	0.43	0.34	0.16	153	69	0.45
C93 (3-1a)	65	0.62	0.11	0.20	260	110	0.42
299-1 (344-32a)	93	0.52	0.44	0.31	66	54	0.82
289-63 (347-13a)	66	0.73	0.76	0.65	82	205	2.50
295-20 (355-XA)	86	0.78	0.88	0.80	75	316	4.21

* The first number represents the original isolation number for each mutant. The number in parenthesis identifies a progeny strain from a cross of the original mutant with either wild-type 740R23-1A or 740R8-1a.

† Numbers in this column are the actual growth in mm for each mutant during the 24-hour period just prior to the shift from 25° to 38°.

‡ 50 ml of Vogel's medium in a 250-ml Erlenmeyer were inoculated with conidia to a final density of 10^6/ml. Flasks were shaken at 190 rpm. Duplicate flasks used for each determination.

The data in Table 1 permit a comparison of growth at permissive and nonpermissive temperatures for wild-type and mutant strains. The cultures were grown in growth tubes at 25° for three successive days, and the distance a culture grew the last 24 hours before being shifted to 38° was compared to the distances grown for the next three days at the nonpermissive temperature. We felt that strains that ceased to grow within three days at 38° could safely be designated ts. By this criterion, the growth of 13 out of 18 mutants clearly was prevented at 38°. Mutants RK3–21, C93, and 299–1 did not stop growing within three days at 38°, but growth of all three strains was significantly retarded at the elevated temperature. Only two mutants, 289–63 and 295–20, grew without progressive decrement at 38° and in this sense were similar to the wild-type controls.

Table 1 also includes the dry weights of mutant and wild-type mycelia grown for 24 hours in liquid medium at 25° and 38°. In addition, the table shows for each strain the ratio of the dry weight at 38° to that at 25°. This ratio provides an index for judging the ts character of mycelial growth in liquid medium. In general, the ratio was low for those strains that also rapidly ceased to grow in

growth tubes at 38°. But there were exceptions. Both A10 and 289–4 essentially stopped growing after one day at 38° in growth tubes; yet, mycelial dry weights were similar for these mutants after 24 hours at 25° and 38° in liquid medium. Interestingly, the ratios were high for mutants 289–63 and 295–20, strains that displayed no progressive decrement in growth tubes at 38°. These data, along with additional data to be presented later, suggest that mutant 295–20 is actually cold sensitive.

Respiration: Respiration in Neurospora can proceed by either of two pathways. Classical respiration involves cytochromes *b*, *c* and aa_3 and is subject to inhibition by agents such as antimycin A or cyanide. However, LAMBOWITZ and SLAYMAN (1971) found that growth of wild-type cells in the presence of these inhibitors or the protein synthesis inhibitor chloramphenicol resulted in the appearance of a respiratory activity that was insensitive to cyanide. This activity, designated the alternate oxidase, is subject to inhibition by SHAM, and is apparently identical to cyanide-insensitive respiration resulting from mutation in the poky strain of Neurospora first observed by TISSIERES, MITCHELL and HASKINS (1953).

The measurement of respiration in the presence and absence of cyanide and SHAM ostensibly permits one to calculate the contributions of the two pathways to the overall rate of respiration. This exercise must be done with caution, however, since BAHR and BONNER (1973) showed that where the two pathways coexist in higher plants, most or all electron flux occurs by means of the cytochrome-mediated pathway in the absence of any inhibitor. Inhibiting the alternate oxidase with SHAM would not substantially affect overall rates of respiration in cells that have a nearly or fully competent cytochrome-mediated pathway. On the other hand, inhibiting the cytochrome-dependent reactions with cyanide would simply shunt electrons over to the previously nonutilized alternate oxidase. The alternate oxidase could have greater electron transfer capacity than the cytochrome-mediated pathway with the result that respiration rates could be even higher in the presence of cyanide than in its absence. Although the wild-type strain of Neurospora normally has very little alternate oxidase activity, EDWARDS, ROSENBERG and MARONEY (1974) showed that this pathway is substantially induced when electron flux through the cytochrome-mediated pathway is diminished by as little as 15 to 20%. The implications are that chemical treatments or mutations that bring about even mild deficiency in the synthesis or function of components in the cytochrome-mediated pathway distal to the branch point will induce the alternate oxidase. This will result in cells that have considerable alternate oxidase capacity, plus an amount of cytochrome-mediated respiration that depends on the severity of chemical inhibition or mutational deficiency. These considerations are important in interpreting the cyanide and SHAM sensitivities of the mutants obtained in the present study.

From Table 2, it can be seen that all mutants except A84, 291-30 and 291-50 show large increases in cyanide-resistant respiration when grown at 38° as compared to wild-type control. Several of these mutants have elevated cyanide-resistant respiration even when grown at 27°. This property is consistent with the screening procedure applied to these mutants during their selection and

TABLE 2

Effect of KCN and SHAM on rates of respiration in mutant and wild-type strains grown at 25° and 38°

Strain	Growth temperature	Respiration* (μ/O_2/hr/mg dry wgt) Control	+ KCN	+ SHAM
wild type	27°	114	9	108
(740R23-1A)	38°	87	8	90
C93	27°	134	11	137
	38°	90	88	62
289-52	27°	123	9	119
	38°	80	66	74
289-56	27°	102	18	96
	38°	79	116	97
289-67	27°	175	37	175
	38°	102	162	73
299-9	27°	130	34	121
	38°	114	170	62
A8	27°	183	44	179
	38°	140	197	76
A13	27°	150	68	166
	38°	119	177	87
A84	27°	128	15	122
	38°	29	10	34
291-30	27°	69	19	85
	38°	38	32	39
291-50	27°	89	20	94
	38°	37	20	37
A10	27°	106	37	99
	38°	93	75	84
RK3-21	27°	134	123	46
	38°	152	188	33
289-4	27°	175	173	77
	38°	133	173	61
297-24	27°	103	129	94
	38°	84	133	85
299-1	27°	170	162	51
	38°	138	149	52
293-18	27°	143	146	137
	38°	113	130	108
295-20	27°	104	139	94
	38°	118	178	109
289-63	27°	138	157	120
	38°	87	97	96

* Respiration measured at 38° regardless of growth temperature. Each figure represents an average of three determinations.

reflects induction of the alternate oxidase, ostensibly as a result of mutation-caused damage or deficiency in one or more components of the cytochrome-mediated respiratory chain.

Mutants C93, 289-67, 299-9, A8, A13, A84, 291-30 and 291-50 show significant decreases in SHAM-resistant respiration when grown at 38° as compared to 27°, and several (RK3-21, 289-4 and 299-1) have similar decreases even when grown at 27°. This result is consistent with the notion that mutation has resulted in moderate to severe damage to the cytochrome-mediated pathway to these strains.

Mutants A84, 291-30 and 291-50 have low rates of respiration in the absence of inhibitors when grown at 38°. These mutants are characterized by relatively low rates of cyanide-resistant and SHAM-resistant respiration when grown at 38°. Presumably these mutants have serious ts defects in cytochrome mediated respiration, combined with only a limited capacity for synthesis of a functional alternate oxidase pathway.

Cytochrome spectra: Table 3 shows the concentrations of cytochromes aa_3, *b* and *c* in mitochondria from wild-type and mutant mycelia grown at 25° and 38°. The strains have been organized into three classes.

Class I includes the wild-type control, along with three of the mutant isolates. Although the three mutants averaged only 60% of the wild-type amount of cytochrome *c*, they all contained normal amounts of cytochromes aa_3 and *b*.

Mutants in Class II, which includes those with a marked deficiency when grown at 25°, had essentially the same spectra properties when grown at 38°. Spectrally, these mutants were not ts, even though certain of them (*e.g.*, 297-24 and 289-4) were in terms of their growth. The group included one mutant deficient in cytochrome aa_3, and five that have low concentrations of both cytochromes aa_3 and *b*. One mutant, RK3-21, was very deficient in cytochrome *c*. To the best of our knowledge, this is the first cytochrome *c* mutant obtained in Neurospora.

Class III consists of eight mutants characterized by ts defects in one or more cytochromes. Mutant A10 was affected only in cytochrome *b*, and a more detailed analysis of this mutant has been published (WEST and PITTINGER 1977). Six mutants were dually deficient in cytochromes aa_3 and *b*, and one displayed a ts deficiency in all three cytochromes.

Cytochrome oxidase and $CoQH_2$-cytochrome c *reductase:* Cytochromes aa_3 and *b* are component parts of the cytochrome oxidase and $CoQH_2$-cytochrome *c* reductase enzyme complexes, respectively. Mutants low in either cytochrome might reasonably be expected to be deficient in the corresponding enzyme. Table 4 shows the specific activities of these two enzymes in mitochondria from wild-type and mutant mycelia grown at 25° and 38°. Data have been arranged in the table according to whether or not enzyme deficiencies were found to be temperature sensitive.

None of the mutants showed a total lack of either enzyme, but consistent differences were repeatedly found. Three of them (299-1, 297-24, 289-4) were partially deficient in one or both enzymes at both 25° and 38°. These same strains

TABLE 3

A classification of mutants based on the relative cytochrome concentrations in mitochondria isolated from normal and mutant strains grown under various conditions of time and temperature

Class and subclass	Strain	Growth conditions (hr at temp.)	Cytochrome concentration (nmoles/mg protein)		
			aa_3	*b*	*c*
Wild-type	740R23-1A)	15@25°	0.35	0.81	1.13
		10@38°	0.38	0.78	1.42
I. Cytochrome content relatively normal compared to wild type	A84	17@25°	0.39	0.89	0.56
	(393-12)	8@25°/9@38°	0.44	0.97	0.80
	291-30	17@25°	0.39	0.84	0.95
	(335-3A)	8@25°/9@38°	0.26	0.74	0.86
	291-50	9@25°†	0.43	0.96	0.60
	(339-6A)	9@25°/12@38°†	0.42	1.01	0.73
II. Deficient in one or more cytochromes @ 25°					
(A) Deficient in cytochrome *c*	RK3-21 (382-9A)	17@25°	0.38	0.69	0.09
(B) Deficient in cytochrome aa_3	299-1 (344-32)	21@25°	0.035	0.86	1.49
(C) Deficient in cytochromes aa_3 & *b*	297-24 (340-49a)	17@25°	0.026	0.16	1.13
	289-4 (322-2A)	17@25°	<0.01	0.19	2.50
	289-63 (370-25)	23@25°	0.06	0.37	1.76
	295-20 (355-34A)	21@25°	0.02	0.17	1.31
	293-18 (326-13)	19@25°	0.16	0.56	1.32
		10@25°/9@38°	0.12	0.35	1.11
III. ts deficiencies in one or more cytochromes					
(A) Deficient in cytochrome *b*	A10 (cyb-3) (301-8A)	16@25°	0.47	0.64 (0.47)*	1.72
		8@25°/8@38°	0.45	0.40 (0.27)*	2.72
(B) Deficient in cytochrome aa_3 & *b*	C93 (3-7a)	14@25°	0.34	0.82	1.10
		14@38°	0.05	0.37	2.96
	289-67 (345-2a)	16@25°	0.38	0.70	1.25
		7@25°/9@38°	0.12	0.17	2.24
	A13 (302-7A)	14@25°	0.35	0.80	1.86
		14@38°	<0.01	0.28	2.75
	289-56 (328-6a)	16@25°	0.38	0.62	1.36
		7@25°/9@38°	0.12	0.39	2.31
	299-9 (343-3-8)	16@25°	0.13	0.45	1.74
		7@25°/9@38°	<0.01	0.23	2.57
	A8 (300-2A)	17@25°	0.10	0.44	1.93
		8@25°/9@38°	0.07	0.10	1.78
(C) Deficient in cytochromes aa_3, *b* & *c*	289-52 (315-6a)	16@25°	0.35	0.63	0.60
		7@25°/9@38°	0.12	0.19	0.13

* Figures in parentheses based on spectra of KCl-washed mitochondria with most cytochrome *c* removed. When cytochrome *c* concentration is high, it leads to overestimations of cytochrome *b* using the equations of WILLIAMS (1964).

† Growth medium supplemented with proteose peptone.

TABLE 4

Specific activities of cytochrome oxidase and $CoQH_2$-cytochrome c reductase in mitochondria from wild-type and mutant mycelium

Strain	Growth conditions (hrs at temp.)	Cytochrome oxidase*	$CoQH_2$-cytochrome *c* reductase*
wild type	13@25°	1.22	2.20
(740R23-1A)	4@25°/9@38°	1.07	2.80
	Enzyme deficiencies not ts		
299-1	22@25°	0.036	4.08
(344-26a)	13@25°/9@38°	0.021	3.83
297-24	16@25°	0.19	0.69
(340-49a)	7@25°/9@38°	0.12	0.60
289-4	19@25°	0.10	0.81
(322-2a)	10@25°/9@38°	0.11	0.90
289-63	17@25°	0.31	0.62
(347-27a)	9@25°/9@38°	0.24	0.76
	Enzyme deficiencies ts		
A10	16@25°	1.16	2.70
(301-8A)	3@25°/13@38°	1.21	0.76
C93	14@25°	1.24	3.79
(3-7a)	14@38°	0.23	2.75
289-67	16@25°	0.93	2.00
(345-2a)	7@25°/9@38°	0.11	0.34
289-52	13@25°	1.23	1.64
(315-6a)	4@25°/9@38°	0.33	0.43
A13	13@25°	1.06	3.04
(302-7a)	13@38°	0.23	0.49
A84	15@25°	1.14	2.28
(303-8A)	7@25°/9@38°	0.62	0.94
291-30	17@25°	0.99	3.14
(335-3A)	7@25°/9@38°	0.43	1.67
289-56	15@25°	1.24	2.68
(324-7A)	7@25°/9@38°	0.29	1.35
299-9	15@25°	0.91	1.50
(343-3-3)	7@25°/8@38°	0.11	0.31
A8	16@25°	0.45	1.14
(300-2A)	7@25°/9@38°	0.10	0.56
295-20	21@25°	0.19	0.55
(355-35a)	12@25°/9@38°	0.34	1.21
	Enzymes relatively normal		
RK3-21	13@25°	0.84	1.98
(382-9A)	5@25°/8@38°	0.61	2.80
293-18	19@25°	0.73	2.78
(326-13A)	7@25°/12@38°	0.80	2.80
291-50	19@25°	0.80	2.72
(339-6A)	12@25°/19@38°	0.79	3.19

* Specific activities expressed as micromoles cytochrome *c* oxidized or reduced per minute per milligram protein.

also showed non-ts deficiencies in the corresponding cytochromes (Table 3). Eleven of the mutants apparently had ts deficiencies in enzyme activity. In one, A10, the defect was limited to $CoQH_2$-cytochrome *c* reductase. Both enzymes were affected in other mutants. Nine of these mutants (291-30 and A84 were the exceptions) also displayed corresponding ts deficiencies in their cytochrome spectra. Both exceptions were moderately ts with respect to cytochrome oxidase and $CoQH_2$-cytochrome *c* reductase activities, but their cytochrome concentrations were essentially normal. Once again, the enzyme data were consistent with the possible cold-sensitive nature of mutant 295-20. Specific activities for both enzymes were significantly higher in cultures grown at 38° than in those grown at 25°.

Enzyme activities of three mutant strains were similar in cultures grown at 25° and 38° and were not substantially different from those of the wild-type strain. One of the mutants, RK3-21, had very little cytochrome *c*. That deficiency, however, would not be expected to have an effect on the *in vitro* activity of either enzyme. Another mutant with normal enzyme activities, 293-18, did appear to be moderately deficient in cytochromes *a* and *b*. Finally, 291-50 had normal enzyme activities and cytochrome concentrations. Though the mutant is decidedly ts with respect to growth, the basis for this characteristic is not yet apparent.

ATPase: Considering the large number of different polypeptides involved in the ATPase complex, we are hopeful that preliminary results with mutants with reduced ATPase activity at the nonpermissive temperature were indicative of mutations in this group of proteins. However, comparing mitochondrial ATPase activity of wild type and mutants proved to be more difficult than we anticipated. Unlike cytochrome oxidase and $coQH_2$-cytochrome *c* reductase, whose specific activities change relatively little, ATPase activity in wild type was found to decline five- or six-fold during the exponential phase of growth. Not only declining ATPase activity during the first 24 hours of growth at 25°, but also reduced activity at 38° during early stages of growth in wild type made it difficult to attribute real significance to low ATPase activity of mutants at either temperature. The interpretation of low ATPase activity was further complicated by the fact that the mutants 293-18 and 291-30, which had low ATPase activity when grown at 38° (Table 5), were also slow growers, especially at the nonpermissive temperature. Thus, in order to get enough mycelia for analysis, mutant cultures were invariably chronologically older than the respective wild-type controls. Furthermore, it was difficult to verify that two cultures of different chronological ages were of similar "physiological age." Because of such considerations, the possible involvement of the ATPase could not be ascertained directly from specific enzyme activities.

On the other hand, several mutants were unique in having mitochondrial ATPase activities that were low or resistant to inhibition by oligomycin (Table 5). ATPase activity was observed to be relatively high in mitochondria from 25°- and 38°-grown cultures of C93, but the mutant was unique when grown at 38° because at that temperature the enzyme was resistant to inhibition by oligomycin. In the case of mutant 293-18, the differences in enzyme activity at the

TABLE 5

Specific activity of ATPase and oligomycin inhibition in mitochondria isolated from wild-type and mutant mycelia grown under various conditions of time and temperature

Strain	Growth conditions (hr at temp.)	ATPase specific* activity (μmoles/min/mg protein)	% Inhibition by oligomycin
wild type	12@25°	1.08	91
(74-OR23-1A)	15@25°	0.78	89
	18@25°	0.32	80
	21@25°	0.26	65
	9@38°	0.64	86
	13@38°	0.17	48
C93-3-7a	14@25°	1.15	90
	16@38°	0.74	−7
293-18	20@25°	0.73	28
(326-13A)	12@25°/9@38°	0.20	23
291-30	16@25°	0.32	87
(335-3A)	7@25°/9@38°	0.32	90

* All activities in table based on spectrophotometric assay coupled to NADH oxidation.

two temperatures were not significantly different from wild type, but the partial resistance to oligomycin when cultures were grown at either 25° or 38° was not observed in other mutants or in the wild-type controls.

DISCUSSION

Filtration enrichment and inositol-less death both proved to be highly satisfactory methods for selecting respiratory-deficient mutants of Neurospora, the incidence of cyanide resistance among confirmed ts mutants being 21% with each procedure. A higher proportion of ts isolates were obtained by using isositol-less death, but the yield of such mutants with filtration enrichment might be improved with longer incubation time at the elevated temperature. Getting rid of the *inl-t* mutant proved to be a troublesome procedure that can be avoided by using the filtration enrichment method.

A gratifying aspect of the study was the wide variety of respiratory-defective mutants that the "cyanide resistance" step enabled us to isolate. On the other hand, a disappointing aspect of this work was our failure to recover, at the nonpermissive temperature, mutants that are totally deficient in cytochrome oxidase, $CoQH_2$-cytochrome *c* reductase, ATPase, or cytochrome-mediated respiration. Two possibilities should be considered. Because TZAGALOFF, AKA. and NEEDLEMAN (1975) recovered mutants in yeast that had normal mitochondrial protein synthesis, yet showed complete lack of activity in cytochrome oxidase, $CoQH_2$-cytochrome *c* reductase or ATPase, one might conclude that our failure to find such mutants constitutes evidence that an intact respiratory chain is indispensible for growth in Neurospora. However, it is conceivable that, in our study, mutants completely deficient in some component of the cytochrome-mediated pathway were induced but passed over in the second stage of selection. Presump-

tive ts isolates that either grew or formed conidia poorly at 25° were not studied further. Perhaps mutants with total dysfunction or lack of some respiratory component at 38° were still so impaired at 25° as to be discarded. SLAYMAN *et al.* (1975) observed slow growth with a strain of Neurospora in which cytochrome-mediated respiration was completely blocked by mutation and antimycin. Apparently, ATP generated through substrate-level phosphorylation and, at Site I, coupled to the alternate oxidase provides sufficient energy to sustain some growth. These investigators concluded that mutants of Neurospora totally lacking one or more components of the respiratory chain should still be viable. Obviously, this is one of the questions we had hoped to answer with the use of conditional lethals, but because so few mutants in this study directly affected the respiratory chain, the question remains unanswered.

Among the mutants that have been isolated and partially characterized in this study, most are pleiotropic, with multiple changes in cytochrome spectrum and in mitochondrial enzyme activities. However, there are some exceptions. Spectral measurements showed 299-1 to have a greatly reduced content of cytochrome aa_3, but normal amounts of cytochromes *b* and *c*. Consistent with these findings, 299-1 also has the lowest cytochrome oxidase activity of any of the mutants. Again on the basis of spectral measurements, RK3-21 has virtually no cytochrome *c*, but a normal content of cytochromes aa_3 and *b*. It is the only cytochrome *c* mutant so far reported in Neurospora, although such mutants have been described in yeast by SHERMAN *et al.* (1970). Two special properties of 299-1 and RK3-21 remain to be accounted for. First, both were isolated as ts mutants and, after backcrossing, continued to show ts growth in growth tubes and in liquid culture. Spectral measurements showed them to be highly abnormal even at 25°, however. Second, although 299-1 and RK3-21 possess gross spectral deficiencies in cytochromes aa_3 and *c*, respectively, leading to the prediction that they should virtually lack respiration *via* the cytochrome chain, the reductions actually measured in SHAM-resistant respiration were only partial. It would appear that we must seriously consider the possibility that, in both cases, the structure of the cytochromes may be altered in such a way as to change drastically their spectral properties without having a parallel effect on catalytic functions.

Over half of the mutants (289-4, 289-56, 289-63, 289-67, 295-20, 297-24, 299-9, A8, A13 and C93) have concurrent deficiencies in cytochromes aa_3 and *b*, thereby resembling the extranuclear poky mutant. Cytochrome defects in poky follow from abnormal assembly of mitochondrial ribosomes (RIFKIN and LUCK 1971), which in turn may be due to a defect in a ribosomal protein synthesized within the mitochondrion (LAMBOWITZ, CHUA and LUCK 1976). It is of interest that not only poky, but other so-called Class I extranuclear mutants (BERTRAND and PITTENGER 1972), have abnormal ribosomal-assembly phenotypes (COLLINS *et al.* 1979). Since cytochrome aa_3 and *b* both have components coded by extranuclear genes, multiple deficiencies in the respiratory chain would be the expected consequences of nuclear or extranuclear mutations impairing any aspect of mitochondrial protein synthesis. Components that could possibly be

affected include ribosomal RNA and proteins, tRNAs, amino acid activating enzymes and other enzymes accessory to mitochondrial protein synthesis. COLLINS, LAPOLLA and LAMBOWITZ (personal communication) have now screened all of our mutants low in cytochromes aa_3 and *b* for possible deficiencies in mitochondrial ribosome assembly and have found that all such mutants, except extranuclear C93, have such defects. For example, mutant 297-24 is deficient in the small ribosomal subunit in both temperatures, whereas 289-56 shows the small subunit deficiency only at 37°. Mutant 289-4 has a large ribosomal subunit deficiency and a large RNA subunit deficiency at 25° and presumably at 38° as well. Two other mutants, A8 and 289-63, show variable small subunit deficiencies. A13 is now known to have a large subunit deficiency, but 289-52, which is ts for all three cytochromes, has normal assembly of the ribosomes. A more detailed analysis of some of these mutants has recently appeared in press (COLLINS *et al.* 1979).

Two isolates, 289-67 and 299-9, which are probably alleles based on negative complementation data, are both ts in subunit assembly and have similar phenotypes that include the appearance of unusual RNA precursors at the nonpermissive temperature. More recent work by MANELLA *et al.* (1979) has shown that these mutants are defective in their excision of the intervening sequence leading to the production of 32S RNA.

Strain 295-20 may warrant separate classification. In liquid culture, the mutant grows better at 38° than at 25°, as does wild type, and this behavior contrasts with the heat-sensitive nature of the other mutants. It is not clear how this mutant came through the selection process, but it may have been related to its interaction with *inl-t*. While 295-20 is deficient in cytochromes aa_3 and *b* at both temperatures, a type of cold sensitivity is clearly manifested in the activities of cytochrome oxidase and $CoQH_2$-cytochrome *c* reductase. Both enzymes are twice as active in cultures grown at 38°, although they are still significantly below wild-type levels. The LAMBOWITZ group has now shown the mutant to be small subunit deficient at both temperatures, but the deficiency is more severe at 25°. It is of interest that a cold-sensitive mutant effecting ribosomal assembly has also been isolated in *E.* coli (GUTHRIE, HASHIMOTO and NOMURA 1969).

The ATPase complex is another enzyme system where we hoped to be able to detect mutants with enzymatic deficiencies, but for reasons already discussed, we were unable to evaluate critically deficiencies in ATPase activity. Nevertheless two mutants possibly had defective ATPase systems, based on the low susceptibility of their ATPase to oligomycin. Mutant 293-18 had low susceptibility to oligomycin when cultured at either 25° or 38°. The extranuclear mutant, C93, was unique in that it was the only mutant showing a complete loss of oligomycin sensitivity in the enzyme from cultures grown at 38°. More recently LAMBOWITZ (personal communication) has found that C93 is deficient in one of the ATPase subunits.

At the present time we have no good evidence as to the nature of the defects in mutants 291-30, 291-50, A84, or 289-52. All are remarkably ts with respect to growth and all except 289-52 (low for all three cytochromes) have nearly nor-

mal quantities of all cytochromes. In this regard they are similar to the *cn* mutants described by EDWARDS and KWIECINSKI (1973). One possibility is that mutations in these isolates have diminished respiratory functions proximal to cytochrome *b*, but unpublished evidence for some of the mutants is not completely supportive of this notion. The mutants might have a regulatory function, and 291-30 may have a defective ATPase.

Unambiguous identification of the primary defects in many of the mutants discussed here will come only with the complete purification and subunit analysis of the several respiratory enzyme complexes and ATPase, as well as through further studies on mitochondrial ribosome assembly and the auxiliary components of mitochondrial protein synthesis. However, this study has amply illustrated the utility of the selection techniques used for isolating respiratory deficient mutants of Neurospora. Obviously, the large proportion of mutants defective in ribosomal assembly were an added bonus. Furthermore, these mutants together with those derived by other workers now constitute a considerable resource that should substantially enhance genetic and biochemical analyses of how nuclear and extranuclear gene products interact in Neurospora mitochondria. Although the studies in Neurospora lack the sophistication of the current genetic and biochemical studies in yeast, there is nevertheless a real need for parallel studies in organisms that are not facultative anaerobes.

We thank CAROL PEAK for excellent technical assistance. Supported in part by the National Science Foundation Grant No. GB-8694. D. J. WEST was supported by Public Health Service training grant HD-00422.

LITERATURE CITED

BAHR, J. T. and W. D. BONNER, JR., 1973 Cyanide-insensitive respiration II. Control of the alternate pathway. J. Biol. Chem. **248**: 3446–3450.

BEADLE, G. W. and E. L. TATUM, 1945 Neurospora II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Bot. **32**: 678–686.

BERTRAND, H., F. E. NARGANG, R. A. COLLINS and C. A. ZAGOZESKI, 1977 Nuclear cytochrome-deficient mutants of *Neurospora crassa*: isolation, characterization, and genetic mapping. Molec. gen. Genet. **153**: 247–257.

BERTRAND, H. and T. H. PITTENGER, 1969 Cytoplasmic mutants selected from continuously growing cultures of *Neurospora crassa*. Genetics **61**: 643–659. ——, 1972 Isolation and classification of extranuclear mutants of *Neurospora crassa*. Genetics **71**: 521–533.

COLLINS, R. A., H. BERTRAND, R. J. LAPOLLA and A. M. LAMBOWITZ, 1979 Mitochondrial ribosome assembly in *Neurospora crassa*: mutants with defects in mitochondrial ribosome assembly. Molec. gen. Genet. (in press)

EDWARDS, D. L. and KWIECINSKI, 1973 Altered mitochondrial respiration in a chromosomal mutant of *Neurospora crassa*. J. Bact. **116**: 610–618.

EDWARDS, D. L., E. ROSENBERG and P. A. MARONEY, 1974 Induction of cyanide-insensitive respiration in *Neurospora crassa*. J. Biol. Chem. **249**: 3551–3556.

GREENWALT, J. W., D. O. HALL and O. C. WALLIS, 1967 Preparation and properties of Neurospora mitochondria, pp. 142–147. In: *Methods in Enzymology*, Vol. 10. Edited by R. W. ESTABROOK and M. T. PULLMAN, Academic Press, New York.

GUTHRIE, C., H. HASHIMOTO and M. NOMURA, 1969 Structure and function of *E. coli* ribosomes. VII. Cold-sensitive mutants defective in ribosome assembly. Proc. Natl. Sci. U.S. **63:** 384–391.

LAMBOWITZ, A. M., N-C. CHUA and D. J. L. LUCK, 1976 Mitochondrial ribosome assembly in *Neurospora*. Preparation of mitochondrial ribosomal precursor particles, site of synthesis of mitochondrial ribosomal proteins and studies on the mutant. J. Mol. Biol. **107:** 223–253.

LAMBOWITZ, A. M. and C. W. SLAYMAN, 1971 Cyanide resistant respiration in *Neurospora crassa*. J. Bacteriol. **108:** 1087–1096.

LAMBOWITZ, A. M., E. W. SMITH and C. W. SLAYMAN, 1972a Electron transport in *Neurospora* mitochondria studies on wild type and *poky*. J. Biol. Chem. **247:** 4850–4858. ——, 1972b Oxidative phosphorylation in *Neurospora mitochondria*. Studies on wild type, *poky* and chloramphenicol-induced wild type. J. Biol. Chem. **247:** 4859–4865.

LESTER, H. E. and S. R. GROSS, 1959 Efficient method for selection of auxotrophic mutants of Neurospora. Science **129:** 572.

MANELLA, C. A., R. A. COLLINS, M. GREEN and A. M. LAMBOWITZ, 1979 Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **76:** 2635–2639.

NARGANG, F. E., H. BERTRAND and S. WERNER, 1978 A nuclear mutant of *Neurospora crassa* lacking subunit 1 of cytochrome *c* oxidase. J. Biol. Chem. **253:** 6364–6369.

RIFKIN, M. R. and D. J. L. LUCK, 1971 Defective production of mitochondrial ribosomes in the *poky* mutant of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **68:** 287–290.

SCHATZ, G. and T. L. MASON, 1974 The biosynthesis of mitochondrial proteins. Ann. Rev. Biochem. **43:** 51–87.

SHERMAN, F., J. W. STEWART, J. H. PARKER, G. J. PUTTERMAN, B. B. L. AGRAWAL and E. MARGOLIASH, 1970 The relationship of gene structure and protein structure of iso-1-cytochrome *c* from yeast. Symp. Soc. Exp. Biol. **24:** 85–107.

SLAYMAN, C. W., D. C. REES, P. P. ORCHARD and C. L. SLAYMAN, 1975 Generation of adenosine triphosphate in cytochrome-deficient mutants of Neurospora. J. Biol. Chem. **250:** 396–408.

TISSIERES, A., H. K. MITCHELL and F. A. HASKINS, 1953 Studies on the respiratory system of the *poky* strain of Neurospora. J. Biol. Chem. **205:** 423–433.

TZAGOLOFF, A., A. AKAI and R. B. NEEDLEMAN, 1975 Assembly of the mitochondrial membrane system. Characterization of nuclear mutants of *Saccharomyces cerevisiae* with defects in mitochondrial ATPase and respiratory enzymes. J. Biol. Chem. **250:** 8228–8235.

TZAGOLOFF, A., A. AKAI, R. B. NEEDLEMAN and G. ZULCH, 1975 Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cervesiae* with lesions in enzymes of the respiratory chain and the mitochondrial ATPase. J. Biol. Chem. **250:** 8236–8242.

VOGEL, H. J., 1964 Distribution of lysine pathways among fungi: evolutionary implications. Am. Naturalist **98:** 435–446.

WEST, D. J. and T. H. PITTENGER, 1977 A temperature-sensitive mutant of *Neurospora crassa*, deficient in cytochrome *b*. Molec. Gen. Genet. **152:** 77–82.

WESTERGAARD, M. and H. K. MITCHELL, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. **34:** 573–577.

WILLIAMS, J. N., 1964 A method for the simultaneous quantitative estimation of cytochrome *a*, *b*, c_1 and *c* in mitochondria. Arch. Biochem. Biophys. **107:** 537–543.

WOODWARD, V. W., J. W. DEZEEUW and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia followed by filtration and plating. Proc. Natl. Acad. Sci. U.S. **40:** 192–200.

Corresponding editor: C. W. SLAYMAN