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Isolation, Mapping, and Characterization of Trehalaseless Mutants of *Neurospora crassa*

ALFRED S. SUSSMAN, M. KERRY GARRETT, MALCOLM SARGENT,[1] AND SHIH-AN YU[2]

Department of Botany, The University of Michigan, Ann Arbor, Michigan 48104

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Mutant strains of *Neurospora crassa* that lack trehalase and are unable to grow on trehalose were isolated, and the gene (*tre*) was positioned on the right arm of linkage group I. Maltase and β-galactosidase activities are almost identical in *tre*⁻ strains, whereas that of invertase was reduced by more than half and those of acid phosphatase and amylase were somewhat increased. Heterocaryons between standard and trehalaseless strains yield less than one-tenth the activity of the former. In addition, strains with duplications heterozygous for trehalase produce less than 1% of the activity of the standard strain. An inhibitor of trehalase has been found in *tre*⁻ strains; its sensitivity to heat and proteolysis, and its nondialyzability suggest that this substance is a protein. The *mig* gene, which determines the rate of migration of trehalase on acrylamide gels, has been shown to be less than 1 map unit away from the *tre* gene.

The requirement of a heat shock for dormant ascospores of *Neurospora* to germinate has been correlated with their inability to utilize a large endogenous store of trehalose (22, 33). Therefore, knowledge of the mechanism through which trehalase (EC 3.2.1.28, α,α'-glucoside 1-glucohydrolase) is controlled is essential before its role in development can be understood.

An increasing body of data attests to the ubiquity of trehalase in the fungi (32), insects (11), mammals (2), higher plants (12), and bacteria (15). However, neither the functions of this enzyme nor the means whereby it is controlled are well understood, although correlations with developmental events have been noted (13, 19). Control over both conidiation and trehalase synthesis in *Neurospora* is exerted by catabolite repression (14) because spore formation and trehalase activity are restricted in the presence of good carbon sources. Another form of control in *Neurospora* was reported by Metzenberg (25), who claimed that trehalase and invertase are coordinately derepressed in a mutant. Other work on trehalase in *Neurospora* has revealed that at least two electrophoretic forms exist (Yu, Garrett, and Sussman, Genetics, *in press*). Moreover, the formation of these "fast" and "slow" forms of the enzyme is controlled by alleles of a single gene.

[1] Present address: Department of Botany, University of Illinois, Urbana, Ill.

[2] Present address: Department of Biology, Eastern Michigan University, Ypsilanti, Mich.

The present report focuses on yet another aspect of the control of trehalase, for we have been able to isolate trehalaseless mutants whose genetics and physiology will be described below.

MATERIALS AND METHODS

Neurospora strains. The standard strain used was 89601A, an inositolless mutant received from the Fungal Genetics Stock Center (FGSC). All other strains used in these experiments, except when otherwise noted, were obtained from the FGSC as well.

Growth media. Except where stated otherwise, growth was on minimal medium (34) containing 10 mg of the appropriate carbon source per ml. Supplements were provided in the following amounts: amino acids, 0.5 mg/ml; vitamins, 10 μg/ml; purines and pyrimidines, 0.5 mg/ml; and inositol, 50 μg/ml.

Crossing techniques. Crossing tubes were prepared by inserting strips of filter paper (10 by 70 mm) in test tubes (16 by 150 mm) containing 5 ml of the medium of Westergaard and Mitchell (35) modified by increasing the biotin concentration 100-fold. On occasion, 5 ml of corn meal agar, without dextrose (Difco), was used in test tubes of the same size. The protoperithecial parent was inoculated several days before fertilization by hyphal fragments and conidia from the other parent. The medium was appropriately supplemented if either parent was auxotrophic.

The *alcoy* strains were used as recommended by Perkins (28). Trehalaseless recombinants were tested on minimal medium containing trehalose and inositol. Slants were examined for growth after at least 5 days at 37 C.

Growth determinations. Standing cultures were grown at 25 C in 125-ml Erlenmeyer flasks containing 20 ml of medium. After the growth period the myce-

lium was placed over a Büchner funnel and washed three times with distilled water. It was then blotted with paper toweling, dried overnight at 95 C, and weighed.

An alternative method was the use of "bubble cultures" in which 200 ml of medium was dispensed into 250-ml Erlenmeyer flasks which were aerated by sparging with compressed air. The mycelium was harvested as described above for standing cultures.

Enzyme assays. Freshly harvested mycelium was ground in "standard buffer" (0.05 M sodium phosphate, *p*H 5.6) in a cold mortar and pestle with washed and ignited sand. Acetone-dried materials were ground by the same means after the acetone was removed by suction in a vacuum desiccator or by air drying. Extracts were either used immediately or stored at −15 C.

Trehalase activity was determined by a modification of the method of Hanks and Sussman (13) in which 2.0 ml of suitably diluted enzyme extract and 0.5 ml of 0.5% trehalose were mixed in duplicate test tubes (15 by 150 mm) containing 1.3 ml of standard buffer. These were incubated at 37 C for 30 min, after which the reaction was stopped by immersion of the tubes in boiling water for 5 min. Reducing sugar was determined by the Nelson modification of the Somogyi method (27). Readings were made with a Klett-Summerson colorimeter with a no. 54 filter. In some cases, the "Glucostat" reagent (Worthington Biochemical Corp., Freehold, N.J.) was used to determine glucose. Trehalase activity is expressed as micrograms of glucose produced per milligram of protein in 30 min.

Invertase and maltase were assayed by incubating 0.1 ml of extract, 1.9 ml of standard buffer, and 0.1 ml of either sucrose or maltose (100 mg per ml) for 30 min at 37 C. Thereafter, the procedure was the same as that described above for trehalase.

Acid phosphatase was assayed by the method of Georgatsos (9), α-amylase by that of Bernfeld (1), glucose oxidase by that of Gibson et al. (10), aspartate transcarbamylase and ornithine transcarbamylase by that of Davis (3), and β-galactosidase by that of Lederberg (21), as modified by Kirby and Lardy (18).

Ornithine transaminase was determined by the method of Davis and Mora (5), with special attention to *p*H, as emphasized by Davis et al. (4).

Tyrosinase was assayed by the method of Sussman (31) with L-dopa as the substrate.

The protein content of extracts was determined by the method of Lowry et al. (23), with bovine serum albumin as the standard.

Complementation techniques. One milliliter of liquid minimal medium, containing trehalose and inositol, was used in test tubes (10 by 75 mm). One loopful of conidia from each strain was suspended in 1.0 ml of the above medium, and single drops of conidia from each pair of strains to be tested were mixed in a tube of fresh medium. Incubation was carried out at 25 C in the dark. The technique was checked by testing known complementing strains which included *arg-5*$^-$ and *arg-8*$^-$ strains kindly provided by Rowland H. Davis, El a (*pan-2*$^-$,*inv*$^-$) and MLS9-262 (*tryp*$^-$,*bd*$^-$).

RESULTS

Isolation of trehalaseless mutants. A technique described by Sargent and Braymer (29), using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for mutagenesis and inositolless death for selection, was used to obtain mutants. Partial mutants, whose genetic nature is not understood, were selected from strain 89601A and used as the basis for subsequent mutant hunts, as a result of which over 200 putative mutants were obtained. These were grown in minimal medium containing trehalose or fructose, and inositol in standing cultures, and dry weights were determined after 7 days. Of these mutants, 166 did not grow at all in trehalose, 22 were "leaky," and the rest grew like the standard strain, 89601A. A small sample of the best mutants were grown in minimal medium containing fructose and inositol in "bubble cultures," and dry weights and trehalase activity were determined. The data reveal a high proportion of the isolates lack trehalase activity yet grow well on fructose.

Growth of trehalaseless strains on various carbon sources. "Bubble cultures" containing minimal medium, 10 mg of the substance tested per ml, and 50 μg of inositol per ml were inoculated with conidia from mutant strains 39-18a and 39-99a and from the standard strain 89601A, and incubated at 25 C. The doubling time and the lag period (hours to attain a dry weight of 0.2 mg/ml) were calculated at the end of 36 hr and are recorded in Table 1. The best carbon sources for all strains used were glucose and sucrose, as judged by short doubling times and lag periods. Longer lag periods are observed in mannitol, xylose, and glycerol, and no growth occurs on lactose or arabinose. Mutant strains differ most from the standard one in that they cannot grow at all on trehalose, although this sugar is an excellent substrate for the latter. Although the mutants tested grew more slowly than the standard strain and had longer lag periods, these effects may be accounted for by secondary mutations induced by the MNNG.

Activity of other enzymes in trehalaseless mutants. The effect of the trehalaseless condition upon the activity of enzymes other than trehalase was studied by comparing such activities (Table 2) in a standard strain (89601A) and in a trehalaseless one (39-19a). Maltase and β-galactosidase activities were almost identical in the mutant and standard strains. The activity of invertase in the mutant was reduced by more than half, but that of acid phosphatase and amylase was increased.

Tests of dominance in heterocaryons. A heterocaryon was constructed between the genotypes *me-6*$^-$ and *nic-2*$^-$ *tre*$^-$ *al-2*$^-$, where *tre* designates the trehalase locus. These were grown in liquid minimal medium containing trehalose, or sucrose, and inositol, and the amount of growth

TABLE 1. *Utilization of various carbon sources by trehalaseless strains of Neurospora crassa*

Carbon source	Strain	Doubling time	Lag[a]
Glucose	89601A	2 hr, 39 min	6 hr, 39 min
	39-18a	3 hr, 43 min	10 hr, 53 min
	39-99a	4 hr, 28 min	4 hr, 0 min
Trehalose	89601A	3 hr, 21 min	7 hr, 51 min
	39-18a	∞[b]	
	39-99a	∞[b]	
Sucrose	89601A	2 hr, 23 min	6 hr, 30 min
	39-18a	3 hr, 38 min	11 hr, 13 min
	39-99a	4 hr, 41 min	10 hr, 55 min
Lactose	89601A	∞[b]	
	39-18a	∞[b]	
	39-99a	∞[b]	
Mannitol	89601A	5 hr, 20 min	7 hr, 45 min
	39-18a	Not tested	
	39-99a	4 hr, 6 min	33 hr, 48 min
Xylose	89601A	3 hr, 55 min	11 hr, 24 min
	39-18a	4 hr, 28 min	15 hr, 20 min
	39-99a	5 hr, 22 min	28 hr, 30 min
Arabinose	89601A	∞[b]	
	39-18a	∞[b]	
	39-99a	∞[b]	
Glycerol	89601A	6 hr, 57 min	19 hr, 48 min
	39-18a	7 hr, 53 min	28 hr, 30 min
	39-99a	Not tested	

[a] Lag period is calculated as the number of hours taken to attain 0.2 mg of growth per ml.
[b] No measurable growth at the end of 36 hr.

TABLE 2. *Comparison of the activity of various enzymes in standard and trehalaseless strains of Neurospora crassa*

Enzyme	Specific activity		Units used[a]
	Strain 89601A	Strain 39-19	
Invertase	4.0×10^{4}	1.6×10^{4}	Glucose, 1 μg
Maltase	1.1×10^{3}	0.9×10^{3}	Glucose, 1 μg
β-Galactosidase	1.1×10^{-3}	1.2×10^{-3}	*o*-Nitrophenol, 1 mmole
Acid phosphatase	18.3	29.3	*p*-Nitrophenol, 1 nmole
Amylase	0.025	0.128	Maltose, 1 μmole

[a] Per milligram of protein in 30 min.

was determined after 7, 9, and 13 days at 25 C (Table 3). Although growth of the heterocaryon on trehalose was a little slower than on sucrose, the total amount obtained at 13 days was about the same. Furthermore, growth of the component strains was faster than that of the heterocaryon in all cases. Trehalase activity was also determined and is recorded in Table 4. These data reveal that the heterocaryon has less than one-tenth of the activity of the *tre*$^{+}$ strain; by 13 days, all of the activity disappears. Although the enzymatic data, taken together with the use of forcing markers, suggest that a heterocaryon did form, it was important to check the results by another means. Therefore, we further tested dominance by the use of a duplication in the region of the *tre* gene.

Tests of dominance using duplications. The availability of translocation stock T (I; VI) NM103, kindly provided by David Perkins,

TABLE 3. *Growth of a heterocaryon between trehalaseless and trehalase-containing strains of Neurospora crassa*

Genotype	Carbon source	Growth[a]		
		7 days	9 days	13 days
nic-2⁺ me-6⁻ tre⁺ al-2⁺	Sucrose	15.2	17.6	14.8
	Trehalose	12.1	14.0	12.0
nic-2⁻ me-6⁺ tre⁻ al-2⁻	Sucrose	16.2	17.4	13.9
	Trehalose	0.2	0.4	0.4
Heterocaryon	Sucrose	11.1	13.2	9.8
	Trehalose	9.4	11.6	10.0

[a] Growth (milligrams per 30 ml of medium) is the average of three samples.

TABLE 4. *Trehalase activity in a heterocaryon between trehalaseless and trehalase-containing strains of Neurospora crassa*

Genotype	Carbon source	Specific activity[a]		
		7 days	9 days	13 days
nic-2⁺ me-6⁻ tre⁺ al-2⁺	Sucrose	671	761	340
	Trehalose	780	840	412
nic-2⁻ me-6⁺ tre⁻ al-2⁻	Sucrose	0	11	5
	Trehalose	0	0	2
Heterocaryon	Sucrose	40	36	0
	Trehalose	54	51	0

[a] Micrograms of glucose produced per milligram of protein in 30 min.

made it possible to produce duplications heterozygous for the *tre* locus alleles in linkage group IR where, it will be shown later, this gene is located. According to Perkins, strain T (I; VI) NM103 has one breakpoint between *thi-1* and *nit-1* in IR (Fig. 1), the other breakpoint being at the right end of linkage group VI. Consequently, when it is crossed to a strain without the translocation, one-third of the surviving progeny are duplicated for all IR markers distal to the breakpoint.

Strains T (I, VI) NM103A and 39-19a were crossed; 7 segregants were selected, from among 65 obtained, as putative duplications on the basis of their slow growth and mycelial characteristics at 34 C, as described by Perkins (28). These were grown for 7 days on minimal medium containing sucrose and inositol, and the mycelium was ground in cold standard buffer containing 0.005 M mercaptoethanol. The addition of mercaptoethanol was found to be necessary for activity to be demonstrated in the strains with duplications but not in standard strains, nor was any activity demonstrable in strain 39-19 even in the presence of this substance. Before use, the enzyme was dialyzed against 0.001 M mercaptoethanol in standard buffer at 4 C overnight. Assays were performed by use of the "Glucostat" technique to avoid interference with the Somogyi reagent by the mercaptoethanol. The enzyme activities found in the strains with duplications and the parental ones are given in Table 5.

The authenticity of the duplications was tested in two ways. First, crosses were made with strain 89601a, and growth of the progeny was compared visually with that of strains 39-19a (*tre⁻*), T (I, IV) NM103A (*tre⁺*), and 89601a (*tre⁺*). Duplications recognized by their slow growth at 34 C were of two classes. One class looked like the parental type duplication in that they grew poorly on trehalose but normally on sucrose. The other class grew equally well on trehalose and sucrose but not as well as nonduplications, and were therefore considered to be duplicated for the *tre⁺* allele. Both *tre⁺* and *tre⁻* nonduplications also were recovered. The recovery of these four classes confirmed the authenticity of the original segregants selected as duplications.

A second experiment tested for breakdown of the duplications which could result in a sectored mycelium carrying duplicated and nonduplicated markers. Conidial suspensions from each duplication were plated on minimal medium containing 0.1% sucrose and 0.8% sorbose. An inoculum from each of 100 randomly selected colonies was then transferred from these plates to tubes containing minimal medium supplemented with either sucrose or trehalose and incubated at 34 C. Growth was compared visually to that of strains T (I, IV) NM103A (*tre⁺*) and 39-19a (*tre⁻*) after 4 days. All of the isolates showed relatively slow growth on sucrose and growth on trehalose was much less than that on sucrose. The trehalaseless control (39-19a) did not grow on trehalose at 34 C. Thus, these isolates consti-

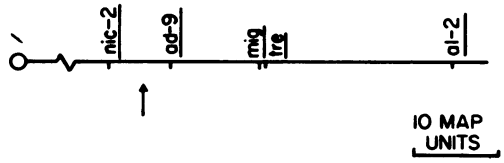

FIG. 1. *Partial map of linkage group I R showing the position of tre as calculated from data in this paper. Arrow designates approximate position of breakpoint on linkage group I for strain NM103 which was used to produce duplications in the region of tre.*

tuted an apparently homogeneous population; if sectoring occurs in these duplications, it must do so on a very small scale since the mean number of nuclei per conidium is approximately 2.5.

Mapping of the trehalaseless gene. The *alcoy* strains described by Perkins (28) were used to position the trehalaseless (*tre*) gene. Crosses of *alcoy* (strain 997) with two different trehalaseless mutants (strains 39-18a and 39-99a) led to tests with markers on linkage group I.

Strain 1216A, an *arg-1*, *leu-3* double mutant with markers on the left arm of linkage group I was used in a cross with a trehalaseless strain (39-18a). The results indicate that a single gene is involved; the order of the genes is leucine, arginine, trehalase, and the latter gene is probably on the right arm of the linkage group. This conclusion was substantiated by the data for a cross between strain 39-18a and a double mutant of *nic-2* and *al-2*. According to the data in Table 6, *tre* is located almost equidistant between *nic-2* and *al-2* (Fig. 1).

Genetic relationship between the tre and mig genes. Evidence presented elsewhere (Yu, Garrett, and Sussman, Genetics, *in press*) indicates that there is a gene (*mig*) in *N. crassa* which determines the rate at which different forms of trehalase migrate electrophoretically. Thus, "fast" and "slow" isozymes of trehalase are determined by the alleles mig^f and mig^s, respectively. Moreover, other experiments have located this gene on linkage group I, probably close to *tre*. Therefore, experiments were performed to discover whether the different migration rates and lack of trehalase are controlled by multiple alleles of a single gene or are determined by separate but closely linked genes.

A means had to be found whereby large numbers of segregants from crosses could be examined quickly. Therefore, crosses were made between strains bearing the following markers: *ad-9*$^-$ mig^s × mig^f *tre*$^-$. The presence of the mig^f allele in the trehalaseless strain was inferred because all such mutants were obtained from strain 89601, which has the fast isozyme of trehalase.

If *tre* and *mig* are nonallelic, it should be possible to identify recombinants producing the fast isozyme of trehalase among the progeny of the above cross. Furthermore, the frequency of *ad-9* prototrophs having the fast isozyme should indicate the order of *tre* and *mig*. Therefore, randomly selected ascospores were activated on minimal medium containing trehalose. The prototrophic segregants were grown in 125-ml Erlenmeyer flasks containing minimal medium with sucrose and inositol, and trehalase was prepared from acetone powders of the mycelium. A rapid means of screening for the different isozymes was developed by the use of antibodies prepared against trehalase from the wall fraction of *Neurospora* mycelium (Sussman, Yu, and Wooley,

TABLE 5. *Trehalase activity in duplications heterozygous for trehalase locus alleles tre*$^+$ *and tre*$^-$

Strain	Specific activity[a]
Duplication	
1	0.8 × 10
2	1.0 × 10
3	1.0 × 10
6	0.9 × 10
7	1.0 × 10
Parent	
39-19 (*tre*$^-$)	$<2.5 \times 10^{-1}$[b]
T (I:IV) NM103 (*tre*$^+$)	6.5×10^3

[a] Micrograms of glucose produced per milligram of protein in 30 min.

[b] Obtained by dividing the threshold of sensitivity of the assay (5 μg of glucose) by the amount of protein contained in the most concentrated assay mixture (20 mg of protein).

TABLE 6. *Results of a cross between a trehalaseless strain of Neurospora crassa (39-18) and a nic-2, al-2 double mutant*

Zygote genotype	*nic-2*$^-$ I *tre*$^+$ II *al-2*$^-$ / *nic-2*$^+$ *tre*$^-$ *al-2*$^+$	No.	Total	Per cent
Parental combinations	*nic-2*$^-$ *tre*$^+$ *al-2*$^-$	92	149	64.2
	nic-2$^+$ *tre*$^-$ *al-2*$^+$	57		
Region I single	*nic-2*$^-$ *tre*$^-$ *al-2*$^+$	25	36	17.2[a]
	nic-2$^+$ *tre*$^+$ *al-2*$^-$	11		
Region II singles	*nic-2*$^-$ *tre*$^+$ *al-2*$^+$	20	43	20.3[a]
	nic-2$^+$ *tre*$^-$ *al-2*$^-$	23		
Regions I and II doubles	*nic-2*$^-$ *tre*$^-$ *al-2*$^-$	2	4	
	nic-2$^+$ *tre*$^+$ *al-2*$^+$	2		

[a] Per cent recombination in regions I and II.

Ind. J. Bot., *in press*). The Ouchterlony double diffusion method was employed with 10 μliters of enzyme, containing about 1 μg of protein, added to each well. Extracts of known fast and slow strains, including those with the *ad-9* background, gave clear-cut differences in Ouchterlony plates (Fig. 2). These differences were eliminated by absorbing the anti-trehalase serum with purified cytoplasmic enzyme. Each plate used for screening by the Ouchterlony test contained two unknown extracts positioned between antigens from strains containing standard fast- and slow-migrating forms of the enzyme. Extracts which indicated the presence of antigens for the fast enzyme, or which gave ambiguous results, were checked by electrophoresis according to the methods of Yu, Garrett, and Sussman (Genetics, *in press*), and all except one such sample were found to have the fast isozyme. When an equivalent number of randomly selected samples, which were identified by the Ouchterlony method as having the slow enzyme were examined electrophoretically, all were found to have this form of the enzyme.

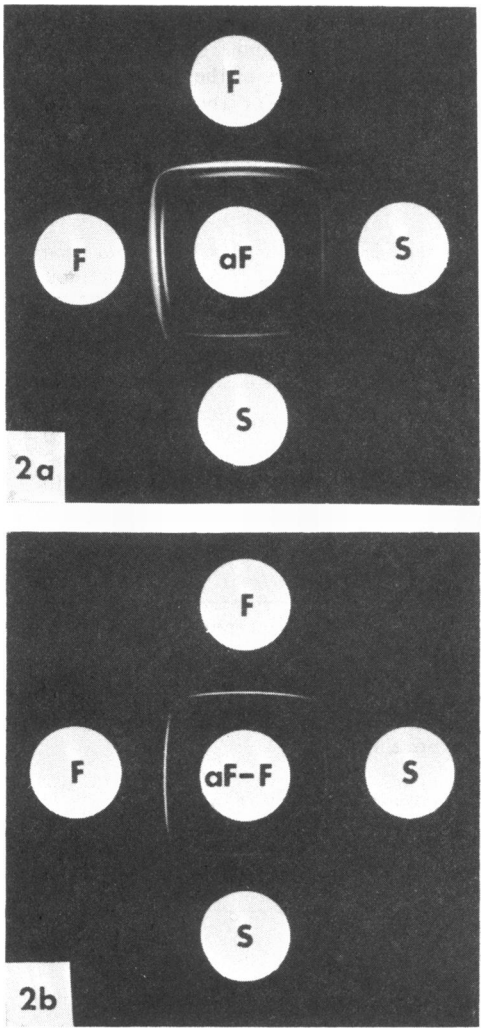

FIG. 2. *Precipitin lines obtained on Ouchterlony plates used to screen extracts from strains of N. crassa having trehalases of different electrophoretic mobility. Peripheral wells: extracts from strains having fast (F) or slow (S) forms of trehalase. Center well of part a, antiserum against trehalase from wall fraction of strain 89601A (aF); center well of part b, antiserum absorbed with purified fast trehalase (aF-F).*

Data for the selection of prototrophs from the cross described above are given in Table 7 where it can immediately be seen that crossing over does indeed occur between the *tre* and *mig* genes. As for the order of the *mig* and *tre* genes, the calculations in Table 7 indicate that *mig* is closer to the centromere than is *tre*. In addition, the distance between *tre* and *mig* is less than one map unit, based upon the isolation of 8,141 viable ascospores, from which 38 recombinants between these loci were obtained among the prototrophs.

Tests for an inhibitor. Although growth on trehalose has been obtained in heterocaryons between trehalaseless and trehalase-containing

TABLE 7. *Observed and expected frequencies of recombinants from the cross ad-9⁻ mig^s tre⁺ × ad-9⁺ mig^f tre⁻ to study the relationship between the tre and mig genes of Neurospora crassa*[a]

Expected frequencies

Electrophoretic type	Order on genetic map[b]	
	ad-9 mig tre	*ad-9 tre mig*
Fast (*ad-9⁺ mig^f tre⁺*)	9.1%	0.99%
Slow (*ad-9⁺ mig^s tre⁺*)	90.9%	99.01%

Results of cross

Electrophoretic type	Determination	Expected no. if	
		mig tre	*tre mig*
	Observed no.		
Fast (*ad-9⁺ mig^f tre⁺*)	38	52.2	5.7
Slow (*ad-9⁺ mig^s tre⁺*)	536	521.8	568.3
	χ^2	4.24	184.87
	p	<5.0 >2.5	<0.001

[a] Only prototrophs were selected from the cross.

[b] Expected frequencies calculated on the assumption that the map distance between the *ad-9* locus and *tre* or *mig* is 10 and that between the latter two loci is 1.

strains, enzyme activity is very low under these conditions. Therefore, a search was conducted for an inhibitor that might control trehalase activity.

Cultures of strain K 49a, a backcrossed trehalaseless mutant, were grown for 7 days at 25 C on minimal medium containing sucrose and inositol. The washed mycelium was ground in cold acetone and air-dried. Samples were suspended in standard buffer overnight at −10 C and centrifuged 20 min at 20,000 × *g*, and the supernatant solution was dialyzed for 5 hr at 8 C against standard buffer. Such preparations were found to be inhibitory to trehalase and were further purified by precipitation in 60 to 80% saturated ammonium sulfate and preparative scale polyacrylamide gel electrophoresis using the pulsed power, vertical gel system produced by Ortec, Inc. (Oak Ridge, Tenn.). The electrophoretically purified inhibitor gave a single protein band in the electrophoresis system described for trehalase isozymes of *Neurospora* (Yu, Garrett, and Sussman, Genetics, *in press*) and gives positive results in the Molisch and biuret tests.

The inhibitor was investigated in the following experiment, the results of which are presented in Fig. 3. Two hundred units of trehalase prepared from strain 89601A by ammonium sulfate precipitation were distributed into each of six sets of test tubes (16 by 125 mm) and incubated at 37 C in the presence of trehalose (1 mg/ml). Controls had no substrate added. The production of glucose was followed by removing portions, stopping the reaction by boiling, centrifuging the samples at 30,000 × *g* for 5 min, and then estimating the glucose by the use of the "Glucostat" reagent. At the end of 27.5 min of incubation, 10 μg of purified inhibitor was added to one of the sets of tubes (Fig. 3, curve A). Another set (Fig. 3, curve B) received 10 μg of purified inhibitor plus trehalose so that the substrate concentration in the assay mixture was increased to 2 mg/ml. One set of controls remained as at the start (Fig. 3, curve C), and the other received additional trehalose (Fig. 3, curve D), as in Fig. 3, curve B. Incubation and periodic assay of the mixtures were continued, and, at 43.5 min, additional trehalose was added to half of the tubes in each set. Some sets (Fig. 3, curve C, and Fig. 3, curve D) showed increased rates upon the addition of trehalose (Fig. 3, curves C′ and D′). Finally, the experiment was concluded at 52 min. The data (Fig. 3) suggest that inhibition occurs rapidly (in <2.5 min) and apparently is not influenced by the addition of trehalose, either at the time of the addition of the inhibitor or subsequently.

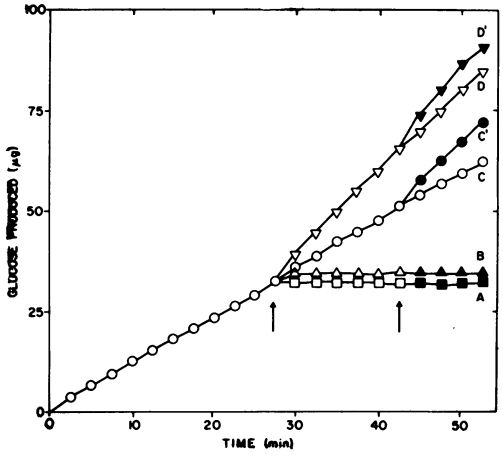

FIG. 3. *Effect of addition of extract from a trehalaseless strain (39-31a) on trehalase activity. Trehalase and its substrate were mixed at zero-time; the extract or trehalose was added at the times designated by the arrows. A, Purified inhibitor (10 mg) was added after 27.5 min; B, as in A, but 1 mg of trehalose per ml was added after 27.5 min; C, no addition; C′, 1 mg of trehalose per ml was added after 43.5 min; D, 1 mg of trehalose per ml was added after 27.5 min; D′, as in D but 1 mg of trehalose was added after 43.5 min. (Solid symbols indicate the effect of additional trehalose at 43.5 min.)*

That all of the trehalaseless mutants obtained produce the inhibitor is suggested by experiments in which 13 randomly selected trehalaseless strains were grown as in the experiment immediately above except that the cultures were harvested after 6 days. Acetone powder extracts were prepared as above; 0.2 ml of the extracts, containing about 300 μg of protein, was mixed with an equal volume of trehalase (1,000 units/ml) and trehalose (1 mg/ml) in the depressions of a spot plate along with controls containing enzyme and substrate alone. A qualitative test of activity was used which employed the change in color of "Testape" (Eli Lilly & Co., Indianapolis) which is based upon the reaction of glucose oxidase in the presence of a chromogen. Considerable inhibition was exerted by all extracts, as judged by the delay in color formation as compared with controls.

A quantitative assay of the inhibitor was performed with seven of the strains tested before by adding enough extract to a mixture of enzyme (500 units/ml) and substrate (1 mg/ml) to give a final concentration of 200 μg of protein per ml. The reaction was carried out for 30 min at 37 C, and the reducing equivalents were determined with the results shown in Table 8. It is clear from these data that approximately equivalent

TABLE 8. *Amount of trehalase inhibitor in trehalaseless mutants of Neurospora crassa*

Assay system	Trehalase activity[a]
Trehalase alone	56
Trehalase + extracts of trehalaseless strains	
39-5a	9
39-9a	10
39-12a	11
39-13a	8
39-19a	9
39-22a	9
39-23a	12

[a] Micrograms of glucose produced in 30 min.

amounts of inhibitor are present in all strains tested.

The specificity of the inhibitor from strain K49a was studied by adding 10 mg of purified inhibitor prepared as above to the appropriate substrate and enzyme. Since neither glucose oxidase, invertase, tyrosinase, acid phosphatase, ornithine transaminase, ornithine transcarbamylase, nor aspartate transcarbamylase were affected by the inhibitor, it appears to be specific for trehalase.

The chemical nature of the inhibitor was studied by determining the effect of several enzymes upon its activity. Thus, 0.1 ml of purified inhibitor in standard buffer was incubated for 3 hr at 37 C with 0.1 ml containing 10 μg of either Pronase, ribonuclease, deoxyribonuclease, or 0.1 ml of standard buffer. After this treatment, 0.1 ml of trehalase and 1 ml of trehalose (5 mg/ml) were added; the reaction mixture was then incubated for 30 min at 37 C whereupon it was stopped by boiling and the reducing sugar determined by the Somogyi method. Controls included samples of trehalase incubated without the inhibitor for 30 min at 37 C with the enzymes mentioned above. As the results in Table 9 indicate, the inhibitor loses its activity only in the presence of Pronase. In another series of experiments, the purified inhibitor was incubated at 65 C for various of time periods, cooled to 37 C, and then mixed with 0.1 ml of trehalase and 1 ml of trehalose (5 mg/ml). A dilution series was used to estimate the amount of inhibitory activity left after heating, as a result of which the inhibitor was found to have a half-life of about 23 min at 65 C.

DISCUSSION

Over 100 mutants lacking trehalase activity and the ability to grow on trehalose have been isolated. No complementation among these has been observed, although insufficient data exist to permit the conclusion that they are identical. All of the mutants tested lacked only trehalase among the eight enzymes studied. In addition, such trehalaseless strains lost the ability to grow on trehalose but not on glucose, sucrose, mannitol, or glycerol, nor did these mutants acquire the ability to grow on lactose or arabinose, substrates that are not used by standard strains of *N. crassa* in "bubble culture."

Although the trehalaseless mutation does not appear to result in the complete loss of any enzyme other than trehalase, the activity of some is affected. For example, invertase activity is reduced by more than half in the mutant. Coordinate changes in trehalase and invertase activities have been observed by Metzenberg (25) and the present results work in this direction, but only partially. An opposite effect was obtained with acid phosphatase and amylase, the activities of which increased in the mutant; as in the case of invertase, no explanation can yet be given for the effect.

Degradation and inhibition of enzymes probably are alternative controls that play an important part in the regulation of enzyme activity that, in turn, controls development. Such appears to be the case in maize where a two-factor inhibitory system is involved in controlling alcohol dehydrogenase (7). Another instance where a distinct selective advantage appears to accrue to the presence of an enzyme inhibitor is that described by Messenguy and Wiame (24) in *Saccharomyces cerevisiae*, wherein wasteful catabolism by the urea cycle is prevented by an arginase which inhibits the activity of ornithine transcarbamylase. Perhaps the protein inhibitor of

TABLE 9. *Effect of certain enzymes upon the activity of the inhibitor of trehalase isolated from strain K 49 of the trehalaseless mutant of Neurospora crassa*[a]

Treatment	Trehalase activity[b]
Trehalase control (no inhibitor)	214
Inhibitor control (untreated inhibitor + trehalase)	108
Pronase + inhibitor + trehalase	172
Pronase + trehalase	190
Ribonuclease + inhibitor + trehalase ..	102
Ribonuclease + trehalase	221
Deoxyribonuclease + inhibitor + trehalase	115
Deoxyribonuclease + trehalase	208

[a] The inhibitor was treated with the enzyme for 3 hr at 37 C, after which it was incubated with trehalase and trehalose for 30 min at 37 C. Results are the average of duplicate samples.

[b] Micrograms of glucose produced in 30 min.

the synthesis of invertase which was recently described (6), the inhibitor of nucleases in *Neurospora* (17), and other inhibitors of proteolytic enzymes (20) and nucleases will eventually be shown to play physiological and developmental roles as well.

The presence of an inhibitor that affects trehalase activity in the hemolymph of blowflies has been reported by Friedman (8). Two factors appear to be involved, including a protein (or proteins) and a metal. However, the role of this inhibitor is uncertain because increased trehalase activity could not be measured under conditions, such as stress, where its substrate is hydrolyzed.

Another inhibitor that is specific for trehalase has been found in approximately equivalent amounts in extracts of seven trehalaseless mutants of *N. crassa*. That this material is a protein is suggested by its retention by dialysis tubing, reaction with protein stains, and its sensitivity to heat and proteolysis. Furthermore, it resists digestion by ribonuclease and deoxyribonuclease. This inhibitor helps to explain the low trehalase activity in heterocaryons between trehalaseless and trehalase-containing strains, which grow on trehalose but less well than does the prototrophic partner. A similar explanation can be offered for the restricted, but significant, amount of trehalase activity found in strains which have duplications covering the region of the *tre* gene on linkage group I. These observations suggest another mechanism through which the dominance of a defective mutant allele over the functional wild-type one might be established. Many examples where mutant alleles override normal ones have been reported from among morphological variants of *Drosophila* (26). Thus, a balance between inhibitor and enzyme might be reached leading to the dominance of the mutant. An alternative has been suggested for glutamic dehydrogenase mutants in *Neurospora* wherein the activity of normal protein monomers is impaired by hybridization with mutant ones (30).

Because of the need to investigate the inhibitor of trehalase in more detail, not much can be said about its function in vivo. However, its discovery may cast some light on the mechanism whereby a heat-shock breaks the dormancy of ascospores of *Neurospora*. The breakdown of a large store of trehalose accompanies the activation of these spores and it has been a mystery as to how trehalase and its substrate coexist in the dormant organism without the latter being hydrolyzed (32). Consequently, it is possible that an inhibitor, which exists in dormant ascospores of standard strains (*unpublished data*), is made ineffective by treatments that break dormancy, thereby releasing trehalase activity.

The *tre* gene is located on the right arm of linkage group I, less than one map unit away from *mig*, which determines the rate of migration of trehalase on acrylamide gels. Such a close association between two genes which affect a single enzyme leads to the question as to which of these genes codes for the inhibitor. One possibility is that the inhibitor is an altered and inactive product of *tre*. On the other hand, *tre* might code for a protein, lacking in the mutants discussed herein, which somehow converts a product of the *mig* gene (the inhibitor?) to active enzyme. These alternatives are the subject of continuing work in this laboratory.

An alternative to the suggestion that the *tre* and *mig* functions are controlled by different genes is that a single gene is involved. Thus, a large gene, or one with a high frequency of interallelic recombinations might be involved. One arm of one protein might be involved with the charge on the exterior of the folded molecule, thereby affecting migration, whereas the other arm affects binding of subunits such that a mutant subunit causes the aggregate to have an altered active site. However, the presence of the inhibitor in both the mutant and the standard strains (unpublished experiments from this laboratory), in addition to the high rate of recombination, argues against this interpretation.

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