Studies on a Temperature-sensitive Irreparable Mutant of *Neurospora Crassa*

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Introduction

Mutants of microorganisms which do not grow on the minimal medium have been obtained by many investigators and used for the study of genetics and biochemistry (Beadle 1957, Fincham 1959). These mutants — biochemical or nutritional mutants — include those which require amino acids, vitamins, purines, pyrimidines, and/or some other growth factors. The mutant which does not respond to any single substance or their combinations but responds only to complex natural materials also belongs to this category.

There is still another type of mutant that does not grow on minimal medium (Horowitz 1950, Horowitz and Leupold 1951, Atwood and Mukai 1953). Horowitz and Leupold (1951) reported that 46% of the temperature-sensitive mutants of *Neurospora crassa* and 23 % of the *Escherichia coli* temperature mutants were irreparable ; that is, they did not grow even on complete media at a certain temperature range where a wild type strain grew well. Atwood and Mukai (1953) obtained 26 non-allelic spontaneous mutants of *Neurospora crassa* in the heterocaryotic form by the technique they devised. Of these only two were reparable ; that is, more than 90 % of the heterocaryon mutants were irreparable, whereas 46 % of temperature-sensitive mutants were found to be irreparable by Horowitz and Leupold (1951). This discrepancy suggests that not all kind of irreparable mutations can be obtained as temperature-sensitive mutants. The temperature-sensitive irreparable mutants, however, have the advantage that they can serve as material for physiological and biochemical analysis.

The present author obtained a mutant which is irreparable at 34°C from a microconidial wild type strain after nitrogen mustard treatment. In this paper the fundamental characteristics of the mutant are described.

Materials and Methods

The temperature-sensitive mutant strain, *b39*, was obtained from direct plating of microconidia of a prototrophic microconidial strain, 8743-21-(13-7)*a* (*pe^mfla*) after

a treatment with 0.5 % methyl-bis (β -chloroethyl) amine hydrochloride in 0.1 M phosphate buffer, pH 6.0. This mutant is the major subject of these studies. Two other temperature-sensitive mutants, *b1* and *b32*, which were used in some of the experiments, were isolated by the same method from the same parent microconidial strain. Both *b1* and *b32* grow well on Fries minimal medium at 23°C. At 34°C, however, *b1* requires acetate for maximal growth. The mutant *b32* has curious growth characteristics at 34°C. It requires adenine supplementation in Fries minimal liquid medium, although glycine supplementation is sufficient in minimal agar medium.

The following markers were used for determining the location of *b39* mutant gene. Four markers, *inos* (37401), *nic-1* (3416), *ad-3* (35203), and *arg-6* (29997) are listed in the paper of Barratt *et al.* (1954). The location of *os* (B135) is shown in Perkins (1956). The morphological mutant *cut* (Kuwana 1953) looks like *os* in its growth form in a test tube (also see Emerson and Emerson 1958), but it was not allelic to *os* as shown in Fig. 1.

Fig. 1. Location of *b39* mutant gene on the first linkage group (mating type chromosome). The location of "*cut*" gene is also shown.

The growth response of the mutant *b39* at 34°C was examined in test tubes containing about 3 ml minimal liquid medium (Beadle and Tatum 1945) and various supplements. Five to ten day old microconidia of the mutant strain cultivated on minimal agar at 23°C were collected and washed twice with distilled water and 10^4 microconidia per ml of medium were pipetted into the test tubes after hemocytometer counts.

Continuous measurements of the growth of the mutants, the parent, and heterocaryons were done on Fries minimal agar medium in the growth tubes described by Ryan *et al.* (1943). Microconidia were collected and washed as described above, and centrifuged down to the bottom of a centrifuge tube. The resulting microconidial paste was put on the agar medium with an inoculum loop. The density of the microconidial suspensions were counted with a hemocytometer, and suspensions of different strains were mixed and centrifuged down for the inoculum of the heterocaryon experiment.

Results

Growth tests of the mutant at 34°C.

While the temperature-sensitive mutant *b39* grows well on Fries minimal medium at 23°C, it does not grow at 34°C even on complete media containing natural nutritional sources such as peptone, yeast extract, Koji extract, meat extract, potato, bread, Miso (soya bean paste) extract, *Neurospora* mycelial extract. Although it is possible that these natural materials might not contain the growth factor the mutant requires, there is also the possibility that the materials contain a substance which inhibits the utilization of a growth factor. Therefore, the following single compounds alone and in some combinations were tested to see whether they might serve as the growth factor of the mutant. None of the substances support the growth of *b39* at 34°C.

These substances are: DL-alanine about 10 and 2 mg / 3 ml medium; L-arginine 10, 2; L-aspartic acid 5, 1; L-asparagine 10, 2; L-cysteine 2; L-cystine 2; Na-L-glutamate 5, 1; glycine 10, 2; L-histidine 5, 1; hydroxyproline 2; DL-isoleucine 2; DL-isoleucine + DL-valine 5: 1, 1: 5; DL-leucine 10, 2; L-lysine 2; L-methionine 10, 2; L-methionine 2 + DL-threonine 2; norvaline 2; ornithine 10, 2; DL-phenylalanine 2; L-proline 2; DL-serine 2; taurine 2; DL-threonine 5, 2; DL-tryptophan 5, 2; L-tyrosine 5, 2; DL-valine 2;

adenine 1; adenosine 1; adenylic acid 1; guanylic acid 2; xanthine 1; thymine 2; cytosine 1; cytidylic acid 1; uracil 2; uridylic acid 1; adenine 1 + uracil 1; adenosine-triphosphate 1.5; yeast nucleic acid 5; thiamine-HCl 1; riboflavine 1; pyridoxine 1; nicotinic acid 1; nicotinamide 1; Ca-pantothenate 1; p-aminobenzoic acid 1; choline 2; inositol 2; folic acid 1; methyl-p-hydroxybenzoate 2; ascorbic acid 25; p-aminobenzoic acid 2 + DL-phenylalanine 2 + L-tyrosine 2 + L-tryptophan 2; Na-succinate 5, 2; Na-acetate 5, 1; Na-butyrate 2; β -indoleacetic acid 1; Na- β -glycerophosphate 10, 2; quinolinic acid 1; fumaric acid 2; kynurenic acid 2; galactose 1; L-arabinose 1; ergosterol 1; sulfathiazole 1; sulfadiazine 1; sulfanilamide 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M.

Neither arabinose 30, 15; galactose 40, 20; lactose 50, 20; sucrose 50, 20; glycerine 70, 30; mannose 30; mannitol 20; L-asparagine 10; Na-glutamate 10; nor Na-succinate 20 supported the growth of *b39* at 34°C as sole carbon sources. Nor KNO_3 10, 5; NH_4NO_3 10, 3; L-asparagine 15; Na-glutamate 5, 2; ammonium tartrate 30, 10; L-asparagine 5 + KNO_3 2 supported the growth as nitrogen sources.

The mutant *b39* was crossed to the osmotic mutant *b11-1A*, and the double mutant was isolated. A protoplast-conidia mixture was obtained from this strain by the method described by Emerson and Emerson (1958). Almost all the substances listed above were tested to determine whether they support the growth of the mutant at 34°C in liquid medium with 8% sucrose, but all the results were negative.

There still remains the possibility that the mutant actually requires some other substances. But such substances must be different from those required by the usual nutritional mutants of microorganisms.

Genetic analysis of b39 mutant gene.

When *b39* was crossed to the other strains, a one to one segregation was observed in each case, both in ordered and random isolations. The mutant therefore originated from a single gene mutation. Results of the genetic analysis are given in Table 1 and

Table 1. Genetic analysis of the mutant *b39*.

Cross	Gene interval	Number of recombinant	Map distance
		Number of total culture	
<i>b39a</i> × <i>inos A</i>	<i>b39</i> —centromere*	12 tetrads/17 tetrads	35 cM
	<i>b39</i> — <i>inos</i>	34 / 63	54 (not linked)
	<i>b39</i> — <i>fluffy</i>	36 / 65	55 (not linked)
	<i>b39</i> —mating type	9 / 47	19
<i>b39a</i> × <i>nic-1 A</i>	<i>b39</i> —centromere*	12 tetrads/23 tetrads	26
	<i>b39</i> — <i>nic-1</i>	46 / 92	50
	<i>b39</i> —mating type	26 / 92	28
<i>b39a</i> × <i>ad-3 A</i>	<i>b39</i> — <i>ad-1</i>	31 / 116	27
	<i>b39</i> —mating type	21 / 114	18
<i>B39a</i> ** × <i>cut A</i>	<i>b39</i> — <i>cut</i>	35 / 113	31

* In the analysis of *b39*—centromere distance the number of tetrads which show 2nd division segregation and total tetrads classifiable were listed.

** *B39a* is a macroconidial mutant isolate from the cross of *b39a* × *nic-1 A*.

Fig. 1. The *b39* mutant gene is located on the left arm of the first linkage group (mating type chromosome) about 30 centi Morgans from the centromere.

Continuous measurement of growth of the mutant at 23°C and 34°C.

At 23°C, the mutant *b39* as well as its parent prototrophic strain grows linearly after a lag period of a day on Fries minimal agar in a growth tube. At 34°C, on the other hand, although the wild-type grows well, the mutant stops its growth after a short residual growth. If the growth tube is transferred to 23°C, the mutant begins to grow at the normal growth rate after a lag period of a day, even after 8 days incubation at 34°C (Fig. 2).

Growth tube cultures of the mutant were then incubated at 23°C and transferred to 34°C (Fig. 3). The transferred cultures at first grew faster than at 23°C, but after a short period the growth rate began to decrease and finally growth ceased. Growth of these cultures continued for 48, 63 and 97 hours at 34°C, when the cultures were transferred after 24, 49 and 72 hours incubation at 23°C respectively (Fig. 3). The longer the period of incubation of the mutant at 23°C, the longer the period of growth at 34°C after the cultures are transferred from 23°C. A period as short as 9 hours at 23°C is sufficient to support growth for about a day at 34°C following the transfer of the culture from 23°C.

Another experiment was done in which the mutant was cultivated for a short period at 23°C, and then transferred to 34°C. After 23, 47, 69, 92 and 120 hours' incubation at 34°C the cultures were transferred back to 23°C. The results are shown

Fig. 2. The growth of the mutant *b39* and the parent wild type strain at 23°C and 34°C on the Fries minimal agar medium in the growth tube. (—•— parent, at 23°C; parent, at 34°C; —○— mutant, at 23°C; mutant, at 34°C; —△— mutant, transferred to 23°C after 3 days' incubation at 34°C; —×— mutant, transferred to 23°C after 8 days' incubation at 34°C

Fig. 3. Cessation of the growth of the mutant *b39* after the culture has transferred from 23°C (—○—) to 34°C after 24 (—×—), 49 (—△—) and 72 (—□—) hours' incubation at 23°C.

Fig. 4. Recovery of the maximal growth rate of the mutant at 23°C (—) after 23 (•), 47 (■), 69 (○), 92 (×) and 120 (△) hours' incubation at 34°C (·····) following preincubation at 23°C (—○—).

in Fig. 4. After 23 hours at 34°C the culture begins to decrease its growth rate. Even if the culture was transferred back to 23°C at this point, the growth rate fell at once and then the maximal growth rate was reached. After 47 hours at 34°C the growth of the culture had almost stopped. If the culture was then transferred back to 23°C at this point, the decreased rate of growth continued for as long as 112 hours. When the cultures were transferred back to 23°C, after the longer period at 34°C, they attained the maximal growth rate after a constant lag period of about 90 hours.

The growth of heterocaryons between b39 and other strains.

The dominance relationships of the mutants *b39*, *b32*, *b1* and their parent strain are determined in heterocaryon experiments. All the strains used here originated from the common parent strain *pe^mfla* and so this system is not complicated by the negative heterocaryon reaction (Garnjobst 1955, Holloway 1955). The three mutants do not grow or grow at a rate only one-twenty fifth of the parent strain at 34°C. Heterocaryons between the mutant strains grow as well as the parent strain (Table 2). Neither *b39*, *b32* nor *b1* inhibited the growth of the other strains in heterocaryons.

Test for growth-inhibiting substances which might be accumulated in the culture medium.

It seemed probable that the mutant could not grow at 34°C, because of the accumulation of some inhibitory substances at this temperature. If the mutant

Table 2. The growth rate of the mutants, their parent strain and heterocaryons between them at 34°C. The growth rates in the linearly growing period in the growth tubes are scored.

Culture		Growth rate (mm/hr)
Homocaryons	<i>pe^mfla</i>	1.9
	<i>b39a</i>	0.0
	<i>b1a</i>	0.04
	<i>b32a*</i>	0.08
Heterocaryons	<i>b39a + bla</i> (10 : 1)	2.4
	" (1 : 1)	2.3
	" (1 : 10)	2.3
	<i>b39a + pe^mfla</i> (10 : 1)	2.2
	" (1 : 1)	2.1
	" (1 : 10)	1.9
	<i>bla + pe^mfla</i> (10 : 1)	2.2
	" (1 : 1)	1.9
	" (1 : 10)	2.0
	<i>b39a + b32a*</i>	2.8
	<i>b1a + b32a*</i>	2.0

* The mutant *b32a* produced little microconidia, so hyphal fragments were used for the inoculations.

Table 3. Effect of the culture filtrates of the mutant and the parent wild type strains on the growth of these two strains.

Culture	Medium	Dry weight of mycelium	
		mg.	Per cent of control
<i>pe^mfla</i>	Fries minimal (control)	68.5	100
	" + <i>pe^mfla</i> medium	88	128
	" + <i>b39a</i> medium	82.5	120
<i>b39a</i>	Fries minimal (control)	33.5	100
	" + <i>pe^mfla</i> medium	35	105
	" + <i>b39a</i> medium	42.5	127

produced some inhibitory substances, its culture medium might inhibit the growth of the mutant especially at an intermediate temperature at which the growth of the mutant in control medium can occur but is somewhat inhibited. This possibility was examined as follows. The mutant and the parent were cultivated in Fries minimal liquid medium for 3 days at 23°C and for the following 4 days at 34°C. The culture media were chilled by a dry ice-methanol mixture, concentrated under reduced pressure, and sterilized by passage through Chamberland bacterial filters. One volume of this sample was mixed with one volume of double strength Fries minimal liquid medium. Microconidia of the mutant and the parent were inoculated and cultivated for 5 days at 26°C, at this temperature the growth of the mutant is about one half of the parent strain. As is seen in Table 3, neither the growth of the mutant nor that of the parent was inhibited by the culture medium of the mutant. This result and the result of the heterocaryon experiment appears to show that the mutant is not inhibited in its growth at 34°C by the accumulation of a product which it produces.

Discussion

There are several possible causes for the appearance of the irreparable mutations. (1) Irreparable mutants might involve multiple-functioned genes (Delbruck's discussion following paper by Bonner 1946, Horowitz 1948). (2) They might require as yet unidentified growth factors. (3) They might be deficient or defective in enzymes not functional in synthetic processes. Most morphological mutants could be of this nature (Sheng 1951). (4) They might require a growth factor which is present in a complete medium but is prevented from penetrating the cells due to an altered cell membrane. (5) They might be unable to retain substances within cells due to altered cell membrane. (6) They might accumulate a specific inhibitor which is not removed by a metabolic product to a point it does not inhibit the growth. Such inhibitor might prevent the penetration of a specific growth factor or an enzyme might be rendered ineffective in its presence.

The single gene mutant *b39* reported in this paper grows as well as the parent wild-type strain on the minimal medium at a temperature around 23°C. But the mutant cannot grow at 34°C even on complete media which contain yeast extract, peptone, Koji extract, meat extract, potato, bread, Miso extract, and/or *Neurospora* mycelial extract. More than sixty single substances and their several combinations were tested to determine whether or not they would support the growth of the mutant, but with negative results. Of course there remained the possibility that the mutant could be made to grow at 34°C by altering the chemical and physical conditions in some way. But such conditions would not be those which are involved in the mutants of microorganisms usually found.

When cultures of *b39* were transferred from 23°C to 34°C, the growth of the mutant was accelerated for short period, after which it began to decrease and finally stopped. The period of the growth at 34°C is longer when the culture was preincubated at 23°C for a longer period (Fig. 3). This may imply that the action of the enzyme systems are favored at the higher temperature but that a certain enzyme or enzyme forming system decomposes rapidly at the higher temperature or that some defect occurs in the permeability of the cell membrane at 34°C.

An interesting growth character is seen in Fig. 4. The cultures of the mutant were incubated first at 23°C and transferred to 34°C, where the growth decreases and finally stops. At various point the cultures were transferred back to 23°C. If the transfer was made just before the growth stopped, the culture continued to grow at a submaximal rate; while if the transfer was made just after the growth stopped, the culture did not grow at all during a lag period. The significance of this difference is not known.

The possibility that this mutant stops its growth at 34°C due to the accumulation of some inhibitory substances in the medium seems to be unlikely; because, as shown in Table 3, the growth of the mutant at an intermediate temperature (26°C) was not inhibited by supplementation of the medium with a culture medium concentrate of the mutant itself,

Summary

(1) Three temperature-sensitive mutants were obtained from a microconidial wild type strain of *Neurospora crassa*. One of them is irreparable at 34°C; that is, it grows as well as the wild type at 23°C, but does not grow on any (including complete) medium so far tested at 34°C. This mutant was the major subject of this report.

(2) The temperature-sensitive irreparable mutant *b39* differs from the parent strain by a single gene mutation. The gene responsible for the irreparable character is located on the first linkage group (mating type chromosome), about 30 cM left of the centromere.

(3) This mutant does not inhibit the growth of the other temperature-sensitive strains in the heterocaryotic condition.

(4) The possibility of the accumulation of inhibitory substances by the mutant is unlikely, because the culture filtrate of the mutant did not inhibit the growth of the mutant at an intermediate temperature.

(5) The growth characteristics of the mutant when the culture was transferred from 23°C to 34°C, and from 34°C to 23°C were determined.

(6) The nature of the defect in an irreparable mutant was discussed.

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