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Characterization of *Neurospora crassa* Mutants Deficient in Glucosephosphate Isomerase

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Two independent mutants of *Neurospora crassa* lacking glucosephosphate isomerase activity (*gpi*) were isolated. These mutants were obtained as double mutants containing the *pp* or T9 mutation in addition to the *gpi* mutation located on linkage group IV; the *pp* mutation caused the inability to form protoperithecium and the loss of ascospore germination, and the T9 mutation caused the alteration in glucoamylase and several growth characteristics. The *gpi* mutants did not grow on fructose but grew on glucose or sucrose. Growth of these mutants on glucose was stimulated by addition of fructose. The *gpi* mutants showed restricted colonial growth on agar media containing glucose in contrast to the normal filamentous growth of the wild-type strain.

Mutants deficient in glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) were successfully isolated from *Escherichia coli* (5, 7), *Salmonella typhimurium* (8), and *Saccharomyces cerevisiae* (12), and several possible roles in metabolism have been suggested for this enzyme. In *Neurospora crassa*, mutants that were altered in the glycolytic enzymes were obtained as morphological mutants, and it was suggested that morphological changes may be caused by the secondary pleiotropic effects of altered metabolic reactions related to cell wall structure (1-4, 11, 14).

This paper describes the isolation and properties of the *gpi* mutants of *N. crassa*, which lack glucosephosphate isomerase activity.

MATERIALS AND METHODS

Organisms. The following strains of *N. crassa* were used: wild-type strains (74A and 3.1a), T9 (T9M150), *gpi1* T9 (T21M3), *gpi2 pp* (T66M37), and *ad-6* (Y234M200).

Growth measurement. To test growth in various media, 0.2-ml portions of conidial suspension (optical density at 620 nm, 0.100) of the various strains were inoculated into 15 ml of liquid media in at least three 100-ml Erlenmeyer flasks and incubated at 25 C. Mycelial mats were harvested after designated times, dried, and weighed. Growth was indicated as the average milligram dry weight of mycelia thus obtained.

Preparation of crude extracts. Mycelia were grown at 25 C for the designated times in a 1,000-ml Roux bottle containing 100 ml of liquid medium. Mycelial mats were harvested on a Buchner funnel, washed several times with deionized water, and ground twice for 5 min with a homogenizer in 0.005 M acetate buffer, pH 5.2. The resulting homogenate was centrifuged at 24,000 × *g* for 60 min, and the supernatant liquid was used as a crude extract.

Enzyme assays. The glucosephosphate isomerase activity was determined by measuring reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) and glucose-6-phosphate formed from fructose-6-phosphate (15). The reaction mixture contained 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4), 4.0 mg of $MgCl_2$, 1 μmol of NADP, 2 μmol of the disodium salt of fructose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and enzyme sample in a total volume of 4.2 ml. The increase in optical density at 340 nm that was brought about by reduced NADP was recorded. Glucose-6-phosphate dehydrogenase and phosphoglucomutase (α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) were assayed by the methods of Brody and Tatum (2, 3).

Determination of protein concentration. Protein concentration was determined by the method of Lowry et al. (10).

Chemicals. Disodium glucose-6-phosphate, disodium fructose-6-phosphate, dipotassium glucose-1-phosphate, tetracyclohexylammonium glucose-1,6-diphosphate, NADP, and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim.

RESULTS

Origin and growth characteristics of the gpi mutants. Two mutants, T21M3 and T66M37, that can not grow in fructose minimal medium were isolated as follows. Mutant T21M3 was obtained from mutant T9, which showed reduced activity of extracellular gluco-

amylase and sorbose resistancy (13). Conidia of strain T9 were treated with ultraviolet light (4 $\times$ 10^6 ergs/cm^2) and subjected to a filtration-enrichment experiment (17; M. E. Case, Neurospora Newslett. **3**:7–8, 1963) in an attempt to select a mutant that does not grow on starch. One mutant, T21M3, which did not grow on starch and fructose, was selected as a possible mutant lacking glucosephosphate isomerase activity. In contrast to the parental strain T9, growth of mutant T21M3 was dependent on the ratio of glucose to fructose in the medium (Fig. 1A and B). The best growth of the mutant was observed when the ratio of glucose to fructose in the medium was 2.0.

From the cross between mutant T21M3 and wild-type 3.1a, the isolates designated *gpi1* were obtained which did not show the T9 mutant phenotype and did not grow on fructose, but grew on glucose. Growth of the *gpi1* isolates on glucose was stimulated by the addition of fructose (Fig. 1C). The *gpi1* isolates showed less growth on glucose or sucrose than that of the original strain T21M3 (Fig. 1C and Table 1).

To isolate *gpi* mutants from wild-type 74A, wild-type conidia were treated with ultraviolet light (10^6 to 4 $\times$ 10^6 ergs/cm^2), X rays (36,000 R), or *N*-methyl-*N'*-nitro-nitrosoguanidine (10 to 20 μg/ml). A minimal medium containing 1.5% fructose was used as the shaking medium in a series of filtration-enrichment experiments (17; M. E. Case, Neurospora Newslett. **3**:7–8, 1963). In the five repeated experiments, only one mutant, T66M37, which showed no growth on fructose (Fig. 1F), was obtained from conidia treated with *N*-methyl-*N'*-nitro-nitrosoguanidine. In contrast to the parental wild-type strain 74A, growth of mutant T66M37 was dependent on the amount of glucose at the later stage of growth (Fig. 1E and F).

TABLE 1. *Growth response of gpi mutants and parental strains in sucrose minimal medium*[a]

Strain	Dry wt of mycelia[b] (mg)
74A	73.9 ± 2.6
T9	73.3 ± 5.8
gpi1 T9	30.1 ± 5.9
gpi1	4.3 ± 1.6
gpi2 pp	35.5 ± 2.4
gpi2	7.7 ± 1.0
gpi2 T9	34.7 ± 5.1

[a] Each strain was grown in at least 10 100-ml Erlenmeyer flasks containing 15 ml of minimal medium supplemented with 1.5% sucrose at 25 C for 5 days.

[b] Average milligrams (dry weight) of mycelia and the standard deviation.

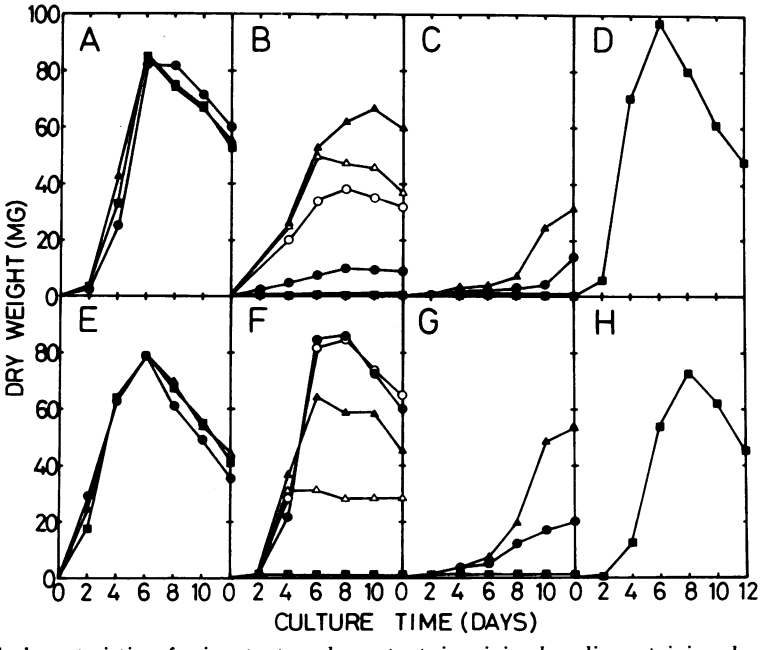

FIG. 1. *Growth characteristics of gpi mutants and revertants in minimal media containing glucose and fructose at various ratios. (A) T9, (B) gpi1 T9 (T21M3), (C) gpi1, (D) R1 T9, (E) 74A, (F) gpi2 pp (T66M37), (G) gpi2, (H) R2 pp. Symbols: ●, 1.5% glucose; ○, 0.4% glucose plus 0.1% fructose; ▲, 1.0% glucose plus 0.5% fructose; △, 0.5% glucose plus 1.0% fructose; ■, 1.5% fructose.*

From the cross between mutant T66M37 and wild-type 3.1a, the isolates designated as *gpi2* were obtained which showed growth characteristics similar to those of the mutant *gpi1* (Fig. 1G and Table 1). The result suggests that mutant T66M37 is a double mutant carrying the mutation *gpi2* and an unknown mutation designated as *pp*, as described below.

The mutants *gpi1* T9, *gpi2 pp*, *gpi1*, and *gpi2* grew on sucrose at similar levels as observed when glucose and fructose were added to the medium (Table 1).

The mutant *gpi1*, *gpi2 pp*, and *gpi2* showed restricted colonial growth on the agar media when the ratio of glucose to fructose in the medium was large, although the wild-type and *gpi1* T9 strains showed normal filamentous growth on these media (Fig. 2). The colony diameter of these mutants became larger and the thickness of the mycelial mat on agar medium became thinner as the ratio of glucose to fructose became smaller (Fig. 2). The diameter of colony did not correlate with dry weight of mycelium in liquid culture, but the thickness of mycelial mat appeared to correlate with it. The reduction of the colony diameter may not be due to the growth inhibition by glucose, but due to the ratio of glucose to fructose, because the diameter of the colony formed on the 0.2% glucose medium without fructose was as small as that formed on 1.5% glucose medium.

Glucosephosphate isomerase activity of the gpi mutant. The activities of glucosephosphate isomerase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase in crude extracts were compared among the wild-type, T9, *gpi1* T9, *gpi2 pp*, *gpi1*, and *gpi2* strains. Crude extracts of the *gpi1* T9, *gpi2 pp*, *gpi1*, and *gpi2* strains had an extremely low activity of glucosephosphate isomerase throughout the growth stages tested, although crude extracts of the wild-type and T9 strains had significant levels of activity of this enzyme (Table 2). The mutants had significant activities of glucose-6-phosphate dehydrogenase and phosphoglucomutase that varied with the culture periods tested.

Crude extract of the strain *gpi1* T9 or *gpi1* was mixed with that of the wild-type strain, but no significant inhibition of the wild-type glucosephosphate isomerase activity was observed. The results suggested that the *gpi1* and *gpi2* mutations are primarily related to the glucosephosphate isomerase activity.

Genetic studies of the gpi mutants. Crosses involving the *gpi* mutants and the *ad-6* mutant indicated that the *gpi1* and *gpi2* mutations are

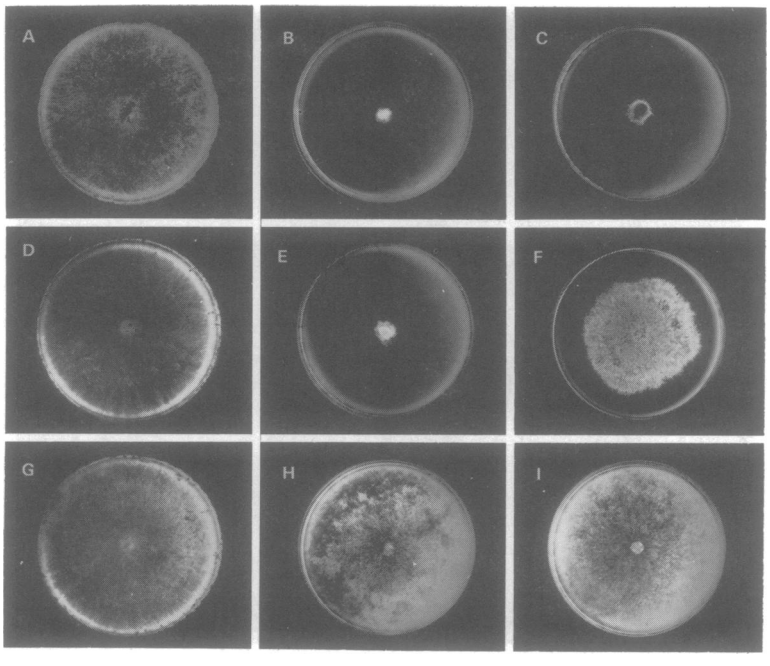

FIG. 2. *Growth characteristics of gpi mutants and wild type on the solid media containing glucose and fructose at various ratios. A drop of conidial suspension of 74A (A, D, and G), gpi1 (B, E, and H), or gpi2 (C, F, and I) was inoculated at the center of agar media and incubated at 25 C for 7 days. (A to C) 1.0% glucose plus 0.5% fructose; (D to F) 0.6% glucose plus 0.9% fructose; (G to I) 0.2% glucose plus 1.3% fructose.*

TABLE 2. *Glucosephosphate isomerase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase activities in crude extracts from gpi mutants and parental strains*

Strain	Culture time (days)	Sp act[a]		
		Glucosephosphate isomerase	Glucose-6-phosphate dehydrogenase	Phosphoglucomutase
74A	3	3.80		
	7	3.00	0.28	0.25
	10	4.25	0.17	0.61
T9	7	2.90	0.37	0.23
gpi1 T9	3	0.01		
	7	0.01	0.51	0.20
gpi1	7	0.00	0.27	0.64
	12	0.00	0.61	0.78
gpi2 pp	3	0.01		
	7	0.01	0.55	0.15
	10	0.02	0.22	0.71
gpi2	7	0.03	0.22	0.38
	12	0.04	0.30	0.58
R1 T9[b]	7	1.25	0.20	0.56
R2 *pp*[b]	7	0.32	0.16	0.15

[a] Crude extracts were prepared from mycelia grown in sucrose minimal medium for 3 to 12 days, and the enzyme activities (units per milligram of protein) were assayed.

[b] Revertants from *gpi1* T9 and *gpi2 pp*.

located 10.0 and 10.8 map units, respectively, from the *ad-6* locus on linkage group IV (Table 3). The cross between mutants *gpi1* and *gpi2* was not successful.

The culture of mutant *gpi2 pp* grown on the crossing medium described by Westergaard and Mitchell (16) produced no protoperithecium as observed under the microscope. The mutants *gpi1* T9, *gpi1*, and *gpi2* produced fertile protoperithecia. When the wild-type strain was grown first on the crossing medium as a protoperithecial parent and the suspension of conidia of the mutant *gpi2 pp* was added 7 days after inoculation, the perithecia were formed. The percentage of germinated ascospores isolated randomly from this cross was 25 to 40%. All 29 asci obtained from this cross had two or more pairs of ungerminated ascospores. About one-half of the germinated ascospores isolated showed the *gpi* mutant phenotype. These results show that mutant *gpi2 pp* is really a double mutant between the *gpi2* mutation and a mutation designated as *pp*, which is related to the formation of protoperithecia and the germination of ascospores.

Double mutant *gpi2* T9, constructed by the cross between mutant *gpi2* and strain T9, showed better growth on sucrose than the single mutant *gpi2* and had the same level of growth as double mutant *gpi1* T9 (Table 1). This indicates that the *gpi1* and *gpi2* mutants may have identical characteristics. The preparation of a double mutant *gpi1 pp* was impossible because no ascospore carrying the *pp* mutation germinated.

TABLE 3. *Linkage data on random segregants from crosses involving the gpi1 and gpi2 mutants*

Strain	*gpi1* × *ad-6*		*gpi2* × *ad-6*	
	Segregant type	No. of isolates	Segregant type	No. of isolates
Parentals	*gpi1* +	127	*gpi2* +	145
	+ *ad-6*	124	+ *ad-6*	136
Recombinants	*gpi1 ad-6*	14	*gpi2 ad-6*	14
	+ +	14	+ +	20

Revertants at the *gpi* locus were obtained by plating conidia of mutant strains *gpi1* T9 or *gpi2 pp* on minimal medium containing 1.5% fructose. Revertants that recovered the ability to reach a level of growth similar to that of the wild-type strain (Fig. 1D and H) also exhibited partially restored glucosephosphate isomerase activity (Table 2) and showed normal growth characteristics on the agar media. This suggests that the *gpi1* and *gpi2* mutations may be single mutations.

DISCUSSION

Two mutant strains deficient in glucosephosphate isomerase activity were obtained as double mutants carrying the *gpi* and T9 or *pp* mutations. Failure to isolate a *gpi* single mutant from a series of filtration-enrichment experiments may be explained by the fact that the *gpi* single mutants, which were obtained from the crosses between the double mutants and the wild-type strain, showed extremely slow growth on any carbon sources tested.

The *gpi* mutants lack glucosephosphate isomerase activity but have the significant activities of glucose-6-phosphate dehydrogenase and phosphoglucomutase. The lack of glucosephosphate isomerase activity may not be due to an inhibitory substance produced in the mutant cells. However, more work will be necessary to demonstrate that the *gpi* mutations are in the structural gene of this enzyme. The *gpi1* and *gpi2* mutations may be allelic, because both mutants lacked the activity of the same enzyme and had identical growth characteristics. Furthermore, both mutations are located at approximately identical regions of linkage group IV.

The *gpi* mutants of *N. crassa* were unable to grow on fructose, but those of bacteria and yeast

are known to grow on fructose (8, 12). Failure to grow on fructose and the low levels of growth on glucose and sucrose in the *gpi* mutants of *N. crassa* might be caused by the toxic effect of sugar metabolites such as sugar phosphate which accumulated because of abnormal ratio of glucose metabolites to fructose metabolites (6, 9).

The growth characteristics of mycelia of the *gpi* mutants on agar media were altered from those of the wild-type strain. A single reversion resulted in the simultaneous restoration of the levels of glucosephosphate isomerase activity and growth characteristics, thus indicating that the limited growth and altered growth characteristics of the mutants are really caused by the same defect. These results are compatible with the results obtained by Brody and Tatum (2, 3), that a colonial mutant and ragged mutant of *N. crassa* are altered in one of the glycolytic enzymes, glucose-6-phosphate dehydrogenase or phosphoglucomutase.

We have indicated that the T9 mutation may be related to cell wall synthesis (13). The *pp* mutation may be related to a complex reaction responsible for the formation of protoperithecia and the germination of ascospores. The considerably improved growth of the double mutants carrying the *gpi* and T9 or *pp* mutations compared with that of the single *gpi* mutant and the differences in growth characteristics observed between *gpi1* T9 and *gpi2 pp* may result from a complex effect concerned with double defects in a cellular metabolism such as the cell wall synthesis. Further work will be required to elucidate the nature of growth properties of these mutants.

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