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Characteristics of a Glycerol Utilization Mutant of *Neurospora crassa*

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A mutant of *Neurospora crassa* able to grow on liquid minimal glycerol medium without evidence of conidiation and with high cell yields has been isolated and shown to be allelic to *ff-1*. The glycerol-specific induction of glycerokinase and glycerol-3-phosphate dehydrogenase was similar in both wild-type and mutant cells, although higher specific activities as well as higher glycerokinase cross-reacting material levels were found in fully induced mutant cells. After growth in minimal glycerol medium there is a significant reduction in wild-type cells of the activities of both pyruvate dehydrogenase and dihydrolipoyl transacetylase. This evidence indicates a relationship between the conditional acetate requirement by wild-type cells grown on glycerol medium and the levels of the pyruvate dehydrogenase complex.

An inducible cytosolic glycerokinase (adenosine 5′-triphosphate:glycerol phosphotransferase, EC 2.7.1.30) (3, 5, 16) and mitochondrial glycerol-3-phosphate (G3P) dehydrogenase (L-G3P:(acceptor) oxidoreductase, EC 1.1.2.1) (3, 4) have been demonstrated in *Neurospora crassa* grown on glycerol as a sole carbon source and are present in sufficient levels to enable *Neurospora* wild-type strains to grow on solidified minimal medium at rates comparable to those measured with fermentable carbon sources (21). In liquid medium, however, only a limited amount of mycelial growth occurs before hyphal propagation ceases and is replaced with extensive conidial proliferation (3), a feature which has hampered utilization of glycerol as an alternative carbon source for certain studies with this organism. Although the conidiation of wild-type strains can be prevented by the growth of cells in a minimal medium containing both glycerol and acetate, the use of such media limits the ability to isolate and study mutants which are specifically defective with regard to their growth on glycerol as a sole carbon source. This paper describes the isolation and biochemical characterization of a mutant of *N. crassa* which is altered in its conidial morphology and has the ability to utilize glycerol as a sole carbon source with high efficiency.

MATERIALS AND METHODS

Neurospora strains. The strains of *N. crassa* employed in this study were obtained from the Fungal Genetics Stock Center (FGSC), Arcata, Calif. The wild-type strain was St. Lawrence strain 74-OR23-1A (74A). The following mutant strains were used in either genetic or biochemical analysis of glycerol utilization: aconidial (*fluffy-fl*), *arg-5*, *arg-13*, *al-2*, *un-5*, and "*alcoy*" [T(I, II) *al-1;* T(IV, V) *cot-1;* (TIII, VI) *ylo-1*] (17). An uncharacterized morphological mutant was isolated from Emerson wild type (FGSC 352) and termed B14. The female sterile (*ff-1*) strain 2318 was obtained from the FGSC. A *glp-1* mutant defective in glycerol utilization, allele 234, was generously provided by H. G. Kølmark (15).

The mutant strain examined in this investigation was obtained as a spontaneous mutant from a culture of 74A grown in a medium containing 1% Tween 80 and 100 mM glycerol and has been backcrossed to 74a four times, without loss of the mutant character. It is designated in this paper as strain 744. In view of the proposal by Perkins and Barratt (Neurospora Newsletter **20**:38, 1973) the gene symbol *glp* will be used to designate mutants altered in their ability to use glycerol. Since two previous mutants have been described as defective in glycerol utilization (15; Nilheden et al., Genetics **74**:s196, 1973; P. Denor and J. B. Courtright, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, H92, p. 111), the glycerol utilization mutant genes studied in this paper are designated *gly-3*$^-$ for defective conidial production on glycerol media.

Growth of cells. Throughout this investigation, cells were grown in appropriately supplemented mineral salts medium of Vogel-Bonner (26), containing either 40 mM acetate, 100 mM glucose, or 100 mM glycerol as the sole carbon source. Amino acids were separately autoclaved and added at a final concentration of 50 to 100 μg/ml. Genetic crosses were performed on cornmeal agar with glucose (Difco). Cell growth was measured as previously described (3).

Enzyme assays. Acetyl-coenzyme A (CoA) synthetase (acetate:CoA ligase [adenosine 5′-monophosphate], EC 6.2.1.1) (8), isocitrate lyase (L_s-isocitrate

glyoxylate-lyase, EC 4.1.3.1) (6), and nicotinamide adenine dinucleotide (phosphate) glycohydrolase (NADase, EC 3.2.2.6) (9) were assayed according to published procedures. Dihydroxyacetone phosphate reductase (L-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8) was determined by measuring dihydroxyacetone phosphate-dependent oxidation of reduced NAD (NADH) in an assay containing 100 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 0.1 μmol of NADH, and 1.0 μmol of dihydroxyacetone phosphate. The rate of NADH oxidation due to NADH oxidase(s) was monitored for 3 min prior to the addition of dihydroxyacetone phosphate. Glycerokinase and G3P were assayed as previously described (3). The pyruvate dehydrogenase complex (PDC) (pyruvate:lipoate oxidoreductase [acceptor acetylating], EC 1.2.4.1, plus acetyl-CoA:dihydrolipoate *S*-acetyltransferase, EC 2.3.1.12, plus NADH:lipoamide oxidoreductase, EC 1.6.4.3) was assayed by the procedure of Harding et al. (7). Dihydrolipoyl transacetylase (acetyl-CoA:dihydrolipoate *S*-acetyltransferase, EC 2.3.1.12) activity was measured in a reaction mixture containing 100 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0), 10 μmol of acetyl phosphate, 0.2 μmol of CoA, 15 μmol of dihydrolipoamide, synthesized from lipoamide (19), and 0.65 U of phosphotransacetylase(acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) in which *S*-acetyl-dihydrolipoamide was determined following the procedure of Reed and Willms (20).

The units of all assays are expressed in terms of nanomoles of substrate consumed or product formed per minute. Specific activity is expressed as units per milligram of protein, where protein concentration was determined with the Folin-Ciocalteau reagent using crystalline bovine serum albumin as a standard (12).

Preparation of extracts. For the assay of glycerokinase and G3P dehydrogenase in cell extracts, mycelia were ground in mortar and pestle as previously described (3).

For the assay of pyruvate dehydrogenase and lipoyl transacetylase, extracts were prepared by the method of Harding et al. (1, 7) or, alternatively, by ammonium sulfate fractionation of crude extracts (27), in which the precipitate was resuspended and dialyzed against 50 volumes of 20 mM potassium phosphate, 0.1 mM thiamine phosphate, and 2 mM beta-mercaptoethanol, final pH 6.8.

Preparation of antibody. Anti-glycerokinase antibodies were prepared by injection of partially purified glycerokinase into rabbits as previously described (4).

Chemicals and reagents. Biochemical reagents were obtained from Sigma Chemical Co.; sucrose was enzyme grade (Schwarz/Mann). All other chemicals were of the highest grade commercially available.

RESULTS AND DISCUSSION

Properties of glycerol-utilizing strains. In previous studies, it has been shown that wild-type strains of *Neurospora* grown on minimal glycerol media in the absence of acetate or long-chain fatty acid supplementation undergo extensive coindiation, and only limited mycelial yields are obtained (Fig. 1). However, a spontaneous mutant isolated in this laboratory (*glp-3$^-$*) and a female sterile derivative obtained from the FGSC both exhibited the ability to utilize glycerol readily as a carbon source as compared to either wild type or the microconidial strain *peach^m* (Table 1). This limited growth was specific for glycerol, since no major differences in cell yield were noted for wild-type cells grown on either acetate, glucose, lactose, or xylose as sole carbon sources.

In addition to the increased mycelial yield on minimal glycerol medium, both strains 744 and 2318 differed significantly from wild-type

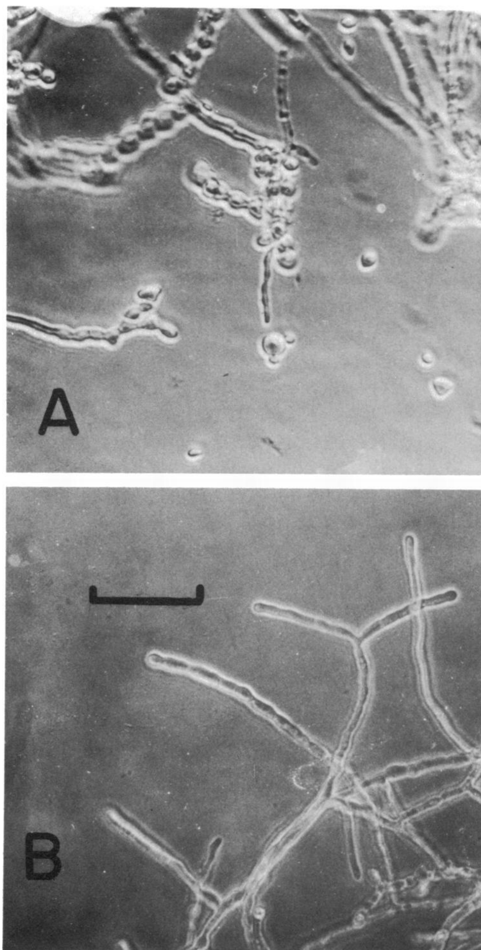

FIG. 1. *Appearance of mycelia of wild type and glp-3$^-$ grown in minimal glycerol medium. Cells were grown in minimal glycerol medium for 24 h at 30 C and were then examined by direct microscopic observation. Cell concentration was approximately 0.6 mg/ml for both 74A (A) and glp-3$^-$ (B). Bar, 50 μm.*

Neurospora in their morphology in that clumps of conidia were present on the surface of minimal sucrose slants and on the sides of the tubes (Fig. 2). This difference in conidial morphology was not altered by any additions to the media tested to date, including Casamino Acids (Difco), yeast extract, peptone, or nutrient broth, and was also not dependent on the carbon source used. These combined features of abundant conidial production on sucrose slants, as well as the high growth rates (Table 2) and yields on minimal glycerol medium, have proved to be useful in the isolation of glycerol-nonutilizing mutants of *Neurospora* (Denor and Courtright, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, H92, p. 111).

TABLE 1. *Cell yields on different carbon sources*[a]

Strain[b]	Carbon source[c]		
	Acetate	Glucose	Glycerol
fluffy	38.0	178.0	67.4
glp-3 (744)	37.2	218.5	216.0
glp-3 (2318)	29.0	210.0	169.2
peach[m]	34.0	205.0	5.0
74A	31.0	278.0	30.1

[a] Cell yields are given in milligrams (dry weight) per 50 ml of minimal medium. Mycelia were harvested after 48 h of growth at 32 C.

[b] All strains were inoculated into 50 ml of minimal medium at a concentration of approximately 10^6 conidia/ml, with the exception of *fluffy*, in which a mycelial mass of approximately 0.1 mg was added directly.

[c] Carbon sources were added to the mineral salts medium to the following final concentrations: acetate, 40 mM; glucose, 100 mM; glycerol, 100 mM.

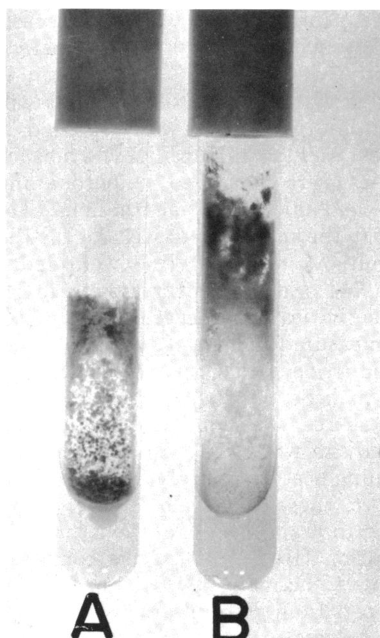

FIG. 2. *Morphological differences in glp-3⁻ and wild-type strains. Cultures of glp-3⁻ (A) and wild type (B) were grown on minimal sucrose slants for 1 week.*

In carrying out the genetic analysis with mutant strain 744, it was soon discovered that, although this strain was isolated on the basis of its glycerol utilization, it was also female sterile under all conditions employed. Conversely, the female-sterile strain 2318, which was isolated on the basis of its female sterility (24), was able to utilize glycerol with high efficiency. The fact that among more than 500 ascospore progeny no recombinants have been obtained that are *glp-3*$^-$ but not female sterile indicates that, if these genes are not allelic, then they must be separated by less than 0.2 centimorgan. However, glycerol utilization associated with the female-sterile strains studied here is not a defining feature, since the other female-sterile strains isolated by Mylyk (13) have been obtained and found to give growth yields on minimal glycerol comparable to strain 74A. Furthermore, glycerol utilization is not a necessary condition for protoperithecium development, since *glp*$^-$ strains (Nilheden et al., Genetics **74**:s196, 1973; Courtright and Denor, Abstr. Annu. Meet. Am. Soc. Microbiol., 1975, H92, p. 111) are able to form protoperithecia. These mutants in *N. crassa* are clearly different from the polyol-defective mutants of *N. tetrasperma*,

TABLE 2. *Mass doubling times of wild-type and glp-3 strains on different carbon sources*

Carbon source[a]	Mass doubling time (h)[b]	
	74A	*glp-3*
Acetate	3.2	3.1
Acetate + glycerol	3.8	2.3
Glucose	2.0	2.8
Glycerol	7.9[c]	4.7

[a] Carbon sources were used at the same concentrations as those given in Table 1.

[b] Mean mass doubling times were calculated from the time-dependent increases in mycelial dry mass. For all experiments, cells were grown on minimal medium at 32 C at cell concentrations of not less than 0.1 mg/ml. Samples of 50 ml were taken at 1-h intervals over a period of not less than 8 h.

[c] Calculations of mass doubling time for 74A on glycerol were based on growth rates in acetate plus glycerol medium, where the cell concentration was greater than 0.7 mg/ml.

which are female sterile and are unable to grow on glycerol, mannitol, and sorbitol (H. Howe, M. Viswanath-Reddy, and S. Bennett, Genetics **77**:232, 1974).

Genetic localization of the gene controlling glycerol utilization. To determine the genetic basis of these morphological strains, which are able to utilize glycerol, strain 744 was crossed to the triply translocated "alcoy" strain (17). Random ascospores were collected, and 58 of 60 *al-1*$^+$ progeny possessed the features of the parental 744 strain, thereby demonstrating that this gene was located on either chromosome I or II. Appropriate crosses to *arg-13 al-2 un-5* (I) and to *arg-5 fl* (II) determined that the mutant character was linked to chromosome II. Further analysis of the progeny derived from the cross *glp(744)* × *arg-5 fl* (Table 3) indicated approximately 20% recombination with *arg-5* and 30% with *fluffy*. As based on these data and the low frequency of *arg*$^-$ *glp*$^-$ *fl*$^-$ recombinants, the location of the mutant gene is placed on chromosome II between *arg-5* and *fl*, about 20 map units from *arg-5*.

Since this map position was almost identical with that previously assigned for the female-sterile feature of strain 2318 (24), it seemed likely that these two mutants represented independently derived alleles of the *glp-3* locus. To establish possible allelism of the mutant character in strain 744 with that in strain 2318, heterokaryons were formed with the genetic composition (*inl*$^-$ *glp*[744] + *his*$^-$ *hlp*$^-$ *glp*[2318]). The resulting heterokaryon grew at 35 C without supplementation, possessed the morphology of both parents, and remained female sterile in attempted crosses. As based on these observations and the similarity in enzymatic characteristics (see below), it is concluded that these features of 744 and 2318 represent independently derived mutations in the *glp-3* gene.

TABLE 3. *Linkage of glp-3 to chromosome II markers*

Genotype[a]	No. of progeny		% Recombination[b] of glp-3 alleles with	
	glp-3$^-$	*glp-3*$^+$	*arg*	*fl*
arg$^+$ *fl*$^+$	78	8	2.9	2.9
arg$^-$ *fl*$^-$	6	74	2.1	2.1
arg$^+$ *fl*$^-$	27	3[c]	1.1	9.8
arg$^-$ *fl*$^+$	39	41	14.1	14.8

[a] Strain *arg-5 fl*$^-$ (*glp-3*$^+$), as the protoperithecial parent, was crossed with *arg-5*$^+$ *fl*$^+$ (*glp-3*$^-$). Ascospores were heat shocked, isolated to individual tubes, and tested for the respective markers by addition of arginine, examination of morphological characters, and/or growth on glycerol.

[b] Percentage of recombination was calculated as the percentage of *glp-3* nonparental combinations per total recovered progeny.

[c] The reason for the low recovery of this progeny class is not understood.

Enzyme activities in glycerol-utilizing strains. In previous studies it was found that *Neurospora* exhibits a form of conditional acetate auxotrophy during growth on minimal glycerol medium, which presumably is due to insufficient amounts of acetyl CoA or a related metabolite(s) rather than to an absence of the enzymes necessary for glycerol dissimilation (3). Since wild-type *Neurospora* apparently decarboxylates a significant fraction of pyruvate to acetaldehyde with resulting ethanol formation during growth on minimal media (2, 23), the possibility existed that strain 744 might synthesize constitutive amounts of one or more enzymes of the glyoxylate cycle, thereby enabling mutant cells to utilize endogenously generated acetate (18). However,a comparison of two key enzymes of the glyoxylate cycle, acetate CoA synthetase and isocitrate lyase, did not reveal constitutive amounts of either enzyme in glucose-grown cells.

Despite the measurable ethanol formation during growth in glucose media (2, 18), wild-type *Neurospora* possesses a mitochondrial pyruvate dehydrogenase complex (7) and therefore must also carry out the oxidative decarboxylation of pyruvate to acetyl CoA. To determine if the activity of the PDC was altered in cells during growth in glycerol medium, wild-type and mutant cell extracts were prepared, and the PDC as well as dihydrolipoyl transacetylase activities were measured in both ammonium sulfate and mitochondrial fractions (Table 4). Although the specific activities for the PDC and dihydrolipoyl transacetylase were reduced in strains 744 and 2318 after growth in glycerol, these activities were several-fold greater than those measured in cells of similarly grown 74A. This reduction in PDC specific activity was only found in cells grown on glycerol as the sole carbon source, since similar reductions were not detected in wild-type cells grown in media containing acetate plus glycerol (Table 4). Furthermore, these low levels of PDC activity are not due to a specific regulation through phosphorylation (10, 11, 27) of the complex, since attempts to activate the residual PDC activity in extracts from glycerol-grown wild-type cells, either by dialysis or by activation through incubation with Mg^{2+} (27), were unsuccessful. Thus, it would appear that the extremely low specific activity found in glycerol-grown wild-

TABLE 4. *Specific activities of the PDC and lipoyl transacetylase in cells grown on different carbon sources*

Strain	Carbon source	Cell fraction[a]	Sp act[b]	
			PDC	LTA
74A	Acetate	AS	10.4	31.5
		M	2.7	89.3
	Acetate + glycerol	AS	14.7	—[c]
		M	2.8	33.2
	Glucose	AS	47.8	45.8
		M	21.4	12.9
	Glycerol	AS	1.5	3.7
		M	1.1	3.2
744	Acetate	AS	2.2	40.4
	Glucose	AS	32.0	53.9
		M	57.3	113.0
	Glycerol	AS	18.8	23.1
		M	10.4	20.3
2318	Acetate	AS	1.8	40.8
	Glucose	AS	59.0	—
	Glycerol	AS	7.3	49.9

[a] Enzymatic activities were determined in either the dialyzed ammonium sulfate fraction (AS) or in a 48,000 $\times$ *g* supernatant of fractionated mitochondria (M) prepared as previously described (11).

[b] Specific activity for PDC or lipoyl transacetylase (LTA) was determined as described in Materials and Methods.

[c] —, Not determined.

type cells is the result of a decreased synthesis of the PDC.

The absence of conidiogenesis during growth on glycerol in mutant strains 744 and 2318 suggested that NADase (NAD glycohydrolase), an enzyme conidiation-defective mutants (22, 25, 28; M. Hochberg and M. Sargent, Neurospora Newsletter **20:**21, 1973), would be similarly absent in the mutant strains during growth on glycerol. Although synthesis of NADase can readily be demonstrated in wild-type cells transferred to minimal glycerol, there was no measurable synthesis of this enzyme in mutant strain 744 during the first 10 h of growth in glycerol medium. This absence is not due to a defect in NADase itself, since measurable enzyme is present after 24 h of incubation and since the structural gene for this enzyme, *nada* (14), is not linked to *glp-3*.

Effect of the glp-3 mutation on glycerokinase and G3P dehydrogenase. In earlier experiments we have shown that the synthesis of both glycerokinase and G3P dehydrogenase is effected by glycerol during growth on minimal acetate medium (3). However, when strain 744 was grown in minimal glycerol, it was found that both specific activities were more than twofold greater than those measured in the

TABLE 5. *Enzyme activities in wild-type and conidiation-defective mutants*

Strain	Carbon source	Sp act[a] (nmol/min/mg)		
		G3PDH	SDH	GK
fluffy	Acetate	68	414	0.3
	Glycerol	858	326	39.8
744	Acetate	56	491	1.2
	Glycerol	609	405	46.9
B14	Glycerol	603	245	84.7
74A	Acetate	48	356	0.4
	Glycerol	372	195	21.6

[a] Specific activities were determined as described (3). Glycerokinase (GK) was measured in the cytosol fraction, whereas glycerol-3-phosphate dehydrogenase (G3PDH) and succinate dehydrogenase (SDH) were measured in mitochondrial suspensions.

wild-type 74A but were not a unique feature of this strain, since similarly high activities were also found in cell extracts of two other mutant strains which fail to conidiate on glycerol medium (Table 5). The glycerokinase activities in these strains were accompanied by a corresponding increase in glycerokinase cross-reacting material, as evidenced by the fact that the same amount of anti-glycerokinase antibody (4) was required for neutralization of 80% of the glycerokinase activity. Since similar increases in the induced levels of both glycerokinase and G3P dehydrogenase occurred in these other morphological variants of *Neurospora*, it is concluded that the degree of induction is a function of the ability of a given strain to grow on glycerol without conidiation rather than a specific genetic factor required for the enhancement of glycerokinase synthesis.

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