Glycerol Uptake Mutants of the Hyphal Fungus *Aspergillus nidulans*

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A new class of glycerol non-utilizing mutants, designated glcC, has been isolated. The glcC gene was mapped in linkage group VI and mutants were found to complement the reference strains glcA2 (linkage group V) and glcB33 (linkage group I) in diploids. The new mutants were unable to grow on glycerol. However, in contrast to the glcA and glcB phenotype these mutants did grow well on dihydroxyacetone and D-galacturonate. By *in vivo* 13C NMR spectroscopy it was shown that the glcC mutant did not take up glycerol but did take up dihydroxyacetone. The latter substrate was converted intracellularly into glycerol which was then catabolized as normal.

**INTRODUCTION**

Glycerol metabolism in filamentous fungi has thus far been studied most extensively in *Neurospora crassa*. Evidence was presented for a catabolic pathway involving phosphorylation to glycerol 3-phosphate and subsequent oxidation of glycerol 3-phosphate in the mitochondria to dihydroxyacetone phosphate (Courtright, 1975). This route was indeed confirmed as glycerol non-utilizing mutants were isolated that lacked either glycerol kinase (gpl) or the mitochondrial membrane-bound flavoprotein glycerol-3-phosphate dehydrogenase (glp2) (Holm et al., 1976; Denor & Courtright, 1978, 1982). However, Viswanath-Reddy *et al.* (1977) and Tom *et al.* (1978) have also suggested that there is an alternative route in which glycerol is utilized by a direct NADP+-dependent oxidation to glyceraldehyde which is similar to the NAD+-dependent oxidation to dihydroxyacetone in *Schizosaccharomyces pombe* and in some other yeasts (Marshall *et al.*, 1985).

In *Aspergillus nidulans* Payton (1978) isolated two classes of glycerol non-utilizing mutants, glcA and glcB, after mutation and filtration enrichment in a glycerol medium. Filtration enrichment in a D-galacturonate medium, however, resulted only in mutants of the glcB genotype (Uitzetter *et al.*, 1986). We have recently found biochemical evidence that in *A. nidulans* glycerol is also catabolized through phosphorylation (glcA) and oxidation (glcB) (J. Visser & coworkers, unpublished results). In this fungus the presence of a NADP+-dependent glycerol dehydrogenase was also established. The latter enzyme is involved in the conversion of dihydroxyacetone into glycerol. The interaction between D-galacturonate breakdown and glycerol catabolism was also previously shown (Uitzetter *et al.*, 1986). The uronic acid is catabolized through glyceraldehyde and pyruvate, the former compound being further metabolized through glycerol. In this study we describe the isolation and characterization of a new class of glycerol non-utilizing mutants (glcC) in addition to the two genotypes already described.

**METHODS**

Strains and mutagenesis. The strains of *Aspergillus* were all derived from the original Glasgow strains (Pontecorvo *et al.*, 1953). The strain used for mutagenesis was H542 (*pabaA1 a1X4 sB43*) from which the glycerol non-utilizing strain H913 was derived. The wild-type control was WG096 (*pabaA1 yA2*). The glycerol non-
Fig. 1. Map of linkage group VI. Map distances ± 1 SD are expressed in centiMorgans. For the map distance nicC to lacA see Clutterbuck (1984).

RESULTS AND DISCUSSION

Identification and mapping of glcC

The strain pabaA1 a1X4 sB43 was mutagenized with 4-nitroquinoline-1-oxide (Bal et al., 1977) and the conidia were plated on medium appropriate for the selection of alcA/alcR mutants (50 mM-glycerol as carbon source with the addition of 2.5 mM-allyl alcohol). Strains that lack alcohol dehydrogenase I (ADHI) either because of a defect in the structural gene, alcA, or a defect in the positively acting regulatory gene, alcR, are unable to metabolize allyl alcohol to its 'toxic' product, acrolein, and are therefore able to grow. A large number of allyl alcohol resistant mutants were obtained and the genetic characterization of mutations in alcA and alcR will be described in detail elsewhere (H. M. Sealy-Lewis, unpublished data), but some properties of these mutations have already been published (Sealy-Lewis & Lockington, 1984). Among a selection of 50 allyl alcohol resistant mutants examined in detail there were two strains carrying alcR mutations, alcR5217 and alcR5233, that were unable to grow on glycerol medium (50 mM-glycerol as carbon source). The strains were outcrossed to yA2 pantoB100 and it was found that the inability to grow on glycerol medium segregated independently from the alcR mutation. Complementation tests were done in diploids between the two glcC mutants, which failed to complement, but showed growth similar to a wild-type control diploid on glycerol medium when tested with either glcA1 or glcB33. The new mutations were designated glcC1 (derived from the alcR5233 strain) and glcC2 (derived from the alcR5217 strain). In a cross between a strain carrying glcC1 and glcC2 there were no progeny that were able to grow on glycerol medium (40 progeny tested). A mitotic haploidization analysis involving a glcC strain and MSD with all the linkage groups marked indicated that the glcC mutation segregated with linkage group VI. Further mapping studies have positioned glcC on the left arm of linkage group VI (see Fig. 1). A four point cross involving glcC, cnxG, nicC and lacA gives the order of markers glcC, cnxG, nicC and lacA. nicC and lacA have been previously mapped (see Clutterbuck, 1984) and this makes glcC the most extreme marker on the left arm of linkage group VI.

Phenotypic characterization of glcC

The growth properties of the glcC mutants were compared with those of the two other known glycerol non-utilizing mutant classes glcA and glcB. The results are summarized in Table 1.
Glycerol uptake mutants of Aspergillus

Fig. 2. Natural abundance $^{13}$C NMR spectra of the polyol resonance region of wild-type *A. nidulans* WG096 (spectra A, C and E) and of the glcC mutant strain H913 (spectra B, D and F). A, B, spectra taken after 18 h of growth on D-glucose (0.1 M); C, D, spectra taken after a 2 h transfer period to glycerol (0.1 M); E, F, spectra taken after a 2 h transfer period to dihydroxyacetone (0.1 M).

Table 1. *Growth characteristics of A. nidulans* glcA, B and C mutants

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>WGO96</th>
<th>glcA</th>
<th>glcB</th>
<th>glcC</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>+++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Glycerol</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Galacturonate</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose/glycerol</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

Whereas *glcA* and *glcB* mutants were unable to utilize dihydroxyacetone, D-galacturonate and glycerol, *glcC* mutants did grow well on the former two substrates. In *A. nidulans* dihydroxyacetone is converted into glycerol by a NADP$^+$-dependent glycerol dehydrogenase activity as indicated previously. Moreover, D-galacturonate and glycerol catabolism have at least one step in common (Uitzetter et al., 1986). Since *glcC* mutants grow well on dihydroxyacetone and D-galacturonate this suggests a defect in a very early step of glycerol catabolism in these new mutants. They were suspected to be defective in their glycerol uptake.

$^{13}$C NMR spectroscopy of *A. nidulans* wild-type and glcC mutant strains

In order to verify whether glycerol uptake was indeed impaired in the *glcC* mutant H913, $^{13}$C NMR spectra of wild-type and of mutant mycelium obtained under different incubation conditions were compared. Mycelia of both strains, grown on glucose for 18 h, were found to have identical natural abundance $^{13}$C NMR spectra (Fig. 2, A and B). Resonances characteristic for the presence of mannitol and erythritol were found in both strains in similar
intensities. After growth on glucose, mycelium was then transferred to a minimal medium containing either glycerol or dihydroxyacetone. After an incubation period of 2 h the spectra of both strains were again compared. In the case of a transfer to glycerol the wild-type spectrum and the glcC spectrum were found to be different (Fig. 2, C and D). In the wild-type the spectral changes are (i) the appearance of glycerol, characterized by resonances at 63.5 p.p.m. (C-1 and C-3) and at 73.2 p.p.m. (C-2), which differ in intensity and (ii) the decrease in intensity of both the mannitol and erythritol resonances. The C-2 and C-3 resonances of erythritol at 73.0 p.p.m. only appear as a shoulder on the resonance at 73.2 p.p.m. of glycerol, which clearly accumulates intracellularly. In the mutant spectrum, however, hardly any spectral change occurred in the 2 h time span (cf. Fig. 2, spectrum B), and in the natural abundance spectra glycerol resonances do not show up.

Upon transfer to a minimal medium containing dihydroxyacetone only glycerol resonances are found in the natural abundance 13C NMR spectra both in the wild-type and the mutant (Fig. 2, E and F). Thus uptake of dihydroxyacetone by the fungus is immediately followed by its reduction to glycerol, which requires NADPH and the presence of a constitutive glycerol dehydrogenase. The other polyol pools have disappeared and thus seem to accommodate the cellular requirement for NADPH by increased catabolism.

Starvation of the mycelium even for prolonged periods of time (6 h or more) does not deplete the polyol pools to the same extent as observed upon incubation with dihydroxyacetone. These results explain why dihydroxyacetone itself is never observed in the mycelial spectra after its uptake. A rapid conversion of this toxic compound into an inert metabolite (glycerol) is required. Since dihydroxyacetone can also arise intracellularly from dihydroxyacetone 3-phosphate this explains the constitutive nature of glycerol dehydrogenase at high levels.

In conclusion our 13C NMR spectra of this glcC mutant show that, when glycerol is given externally, it does not appear in the mycelium. However, when glycerol is generated intracellularly from dihydroxyacetone it can serve as a carbon source leading to normal growth behaviour. These results identify glcC as a glycerol uptake mutant. We have subsequently tested glcC for growth on glycerol over a wide range of glycerol concentrations (50 mM–1 M). No growth at all was found for the mutant even at a 1 M concentration. It thus seems that even at high concentrations diffusion of glycerol is insufficient for growth and that there is an absolute requirement for a specific glycerol transport system.

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REFERENCES


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