Selection and characterization of *Aspergillus nidulans* mutants involved in regulation of glycerol metabolism

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Pathway-specific induction plays an important role in *Aspergillus nidulans* when this fungus is grown on media containing glycerol as carbon source. To obtain mutants altered in regulation of glycerol metabolism, we used a *glcB* mutant strain, which is unable to grow on mixed carbon sources like D-glucose/glycerol as it accumulates G3P. Pseudorevertants, derived from this strain after mutagenesis as able to grow on D-glucose/glycerol, were analysed. In addition to glycerol kinase mutants and glycerol uptake mutants four new classes of mutants were found. Biochemical and genetic data indicate that the *glcH* mutants found are likely to lack a positive factor necessary for full induction of glycerol kinase and glycerol-3-phosphate dehydrogenase activities. The phenotype of a temperature-sensitive *glcI* mutant was found to be influenced by oxygen tension. Elevated levels of intracellular phosphatase were found in a *glcL* mutant. The *glcK1* mutant had slow growth rates and higher levels of phosphofructokinase and fructose-6-phosphate reductase.

Introduction

In *Aspergillus nidulans* glycerol is catabolized by conversion to dihydroxyacetone phosphate (DHAP). After uptake, mediated by the *glcC* gene product, glycerol is first converted into glycerol 3-phosphate (G3P) by glycerol kinase (encoded by *glcA*). G3P is then oxidized to DHAP by a mitochondrially located FAD-dependent G3P dehydrogenase (encoded by *glcB*, *glcD* and *glcG*) (Arst *et al.*, 1990; Hondmann *et al.*, 1991). Dihydroxyacetone (DHA) and D-glyceraldehyde (D-GAD) catabolism is tightly linked to glycerol metabolism in *A. nidulans*. Both substrates are reduced intracellularly to glycerol, DHA by a constitutive NADPH-dependent reductase, and D-GAD by alcohol dehydrogenase I and by a constitutive NADPH-dependent reductase (Hondmann *et al.*, 1991). The two NADPH-dependent reductase activities are attributed to a single enzyme that has been purified and characterized (Schuurink *et al.*, 1990).

Two forms of regulation are found for both the glycerol kinase and the FAD-dependent G3P dehydrogenase activity. In the presence of glycerol, DHA and D-galacturonate, pathway-specific induction occurs,

whereas in the presence of repressing carbon sources such as D-glucose, carbon catabolite repression is found (Hondmann *et al.*, 1991).

A mutant of particular interest for the study of glycerol metabolism is the *glcB33* mutant. This mutant has no G3P dehydrogenase (FAD) activity. As a result, G3P accumulates when mycelium of the mutant is transferred to media containing glycerol or DHA. Due to the accumulation of G3P the *glcB33* mutant is unable to grow on media containing mixed carbon sources, e.g. D-glucose/glycerol or D-glucose/DHA.

We present here a study in which the *glcB33* mutant is used to select pseudorevertants that grow on D-glucose/glycerol. The nature of these pseudorevertants is discussed.

Methods

Materials. G3P dehydrogenase, lactate dehydrogenase, pyruvate kinase, enolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate dehydrogenase, triosephosphate isomerase (all enzymes from rabbit muscle), glucose-6-phosphate dehydrogenase (from yeast), G3P, glycerate 2-phosphate, glycerate 3-phosphate, glycerate 2,3-diphosphate, glyceraldehyde 3-phosphate, phosphoenolpyruvate, erythrose 4-phosphate, fructose 6-phosphate, 6-phosphogluconate, *p*-nitrophenyl phosphate, ADP, ATP, NADH, NADPH, NADP⁺ and NAD⁺ were supplied by Boehringer. D-GAD, DHAP and MTT were from Sigma. DHA was from Serva. D-Galacturonic acid was obtained from Fluka. Sephadex G-25 was obtained from Pharmacia LKB. Other reagents were from Merck and were of analytical grade.

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Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate, G3P, glycerol 3-phosphate; D-GAD, D-glyceraldehyde.

Strains and culture conditions. Strain WG096 (*yA2 pabaA1*) was used as wild-type strain with respect to carbon source utilization. The glycerol non-utilizing strains WG196 (*yA2 pabaA1 glcA1*), WG197 (*yA2 pabaA1 glcB33*), WG372 (*yA2 pantoB100 glcC2*), WG373 (*yA2 pabaA1 glcE1*), WG374 (*yA2 pabaA1 glcF1*), WG380 (*biA1 glcF1*), WG381 (*biA1 glcE1*), *glcD100* (*pabaA1 pantoA10 glcD100*) and *glcG1* (*yA2 pabaA1 glcG1*) were used. As tester strain for linkage group assignment WG202 (*biA1 acrA1 meaB6 pyroA4 pA1 sB3 nicB8 riboB2*) was used. As a tester strain for the assignment of the *glcK* locus on chromosome II, WG362 (*biA1 acrA1 wA7 palcA1 riboE6 thiA4 ileA3 cnxEl6 ygA6*) was used. Genetic techniques were performed according to standard procedures (Pontecorvo *et al.*, 1953; Clutterbuck, 1974). Culture conditions were described previously (Hondmann *et al.*, 1991).

Growth tests were performed on minimal medium solidified with 1.2% (w/v) agar and containing appropriate supplements, as described previously (Uitzetter *et al.*, 1982). Carbon sources (pH 6) were added separately as membrane filter (0.2 µm, Schleicher and Schüll) sterilized solutions. The final concentrations were: glycerol, 100 mM; DHA, 100 mM; D-galacturonic acid, 50 mM; glucose/glycerol, 25 mM/100 mM; glucose, 50 mM; fructose, 50 mM; glucose/DHA, 25 mM/100 mM; glucose/D-galacturonic acid, 25 mM/50 mM; acetate, 100 mM; acetate/glycerol, 100 mM/100 mM. Microaerophilic growth conditions were obtained by incubating test plates in an 'exicator' containing a BBL gas generator envelope for production of a reduced oxygen atmosphere, and which was used according to the manufacturer's instructions.

Mutagenesis. A suspension of approximately 10^8 conidia from strain WG197 (*glcB33*) was irradiated for 1 min with a Philips TUV-lamp at a dose of $120 \text{ J m}^{-2} \text{ min}^{-1}$. This dose provides an optimum balance between the number of mutants that can be expected and background genetic damage (Bos, 1986).

Preparation of cell free extract. Cell free extracts were prepared according to Arst *et al.* (1990).

Enzyme assays and protein determination. Phosphatase activities were assayed in cell-free extracts essentially according to Caddick & Arst (1986). Also a 10 mM-glycylglycine/piperazine buffer was used to measure activities in one buffer system covering the range from pH 4.0 to 10.0. Phosphatase assays were performed at 30 °C.

Hexokinase (EC 2.7.1.1) was assayed in a reaction mixture containing 100 mM-Tris/HCl (pH 7.5), 10 mM-MgCl₂, 0.15 mM-NADP⁺, 0.2 mM-ATP, 1 U glucose-6-phosphate dehydrogenase, 4 mM-glucose. Phosphoglucose isomerase (EC 5.3.1.9) was assayed in 40 mM-triethanolamine (pH 7.6), 8 mM-MgCl₂, 1 mM-NADP⁺, 1 U glucose-6-phosphate dehydrogenase, 2 mM-fructose 6-phosphate. Phosphofructokinase (EC 2.7.1.11) was assayed in 50 mM-HEPES (pH 7.8), 2 mM-MgCl₂, 0.15 mM-NADH, 1 mM-ATP, 10 U G3P dehydrogenase, 10 U triose phosphate isomerase, 10 mM-fructose 6-phosphate. Fructose-1,6-diphosphate aldolase (EC 4.1.2.13) was assayed in 100 mM-triethanolamine (pH 7.6), 10 mM-EDTA, 0.2 mM-erythrose 4-phosphate, 0.15 mM-NADH, 1 U G3P dehydrogenase, 10 U triose phosphate isomerase, 3 mM-fructose 6-phosphate. Triose phosphate isomerase (EC 5.3.1.1) was assayed in 100 mM-triethanolamine (pH 7.6), 0.15 mM-NADH, 1 U G3P dehydrogenase, 4 mM-glyceraldehyde 3-phosphate. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) was assayed in 100 mM-triethanolamine (pH 7.6), 1 mM-ATP, 1 mM-EDTA, 2 mM-MgSO₄, 0.15 mM-NADH, 20 U phosphoglycerate kinase, 6 mM-glycerate 3-phosphate. Phosphoglycerate kinase (EC 2.7.2.3) was assayed in 40 mM-triethanolamine (pH 7.6), 1 mM-EDTA, 0.15 mM-NADH, 1 mM-ATP, 2 mM-MgSO₄, 5 U glyceraldehyde-3-phosphate dehydrogenase, 5 mM-glycerate 3-phosphate. Phosphoglyceromutase (EC 2.7.5.3) was assayed in 100 mM-triethanolamine (pH 7.6), 1 mM-MgSO₄, 0.15 mM-NADH,

0.6 mM-ADP, 0.12 mM-glycerate 2,3-diphosphate, 10 U lactate dehydrogenase, 2 U pyruvate kinase, 2 U enolase, 5 mM-glycerate 3-phosphate. Enolase (EC 4.2.1.11) was assayed in 100 mM-triethanolamine (pH 7.6), 4 mM-MgSO₄, 0.15 mM-NADH, 1 mM-ADP, 20 U lactate dehydrogenase, 3 U pyruvate kinase, 1 mM-glycerate 2-phosphate. Pyruvate kinase (EC 2.7.1.10) was assayed in 100 mM-triethanolamine (pH 7.6), 5 mM-MgCl₂, 2 mM-ADP, 0.15 mM-NADH, 13 U lactate dehydrogenase, 10 mM-KCl, 10 mM-(NH₄)₂SO₄, 5 mM-phosphoenolpyruvate. Fructose-1,6-diphosphatase (EC 3.1.3.11) was assayed in 50 mM-Tris/HCl (pH 8.0), 1 mM-EDTA, 5 mM-MgCl₂, 0.4 mM-NADP⁺, 1 U glucose-6-phosphate dehydrogenase, 1 U glucose-6-phosphate isomerase, 2 mM-fructose 1,6-diphosphate. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in 50 mM-Tris/HCl (pH 8.0), 5 mM-MgCl₂, 0.4 mM-NADP⁺, 5 mM-glucose 6-phosphate. 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was assayed in 50 mM-glycylglycine (pH 8.0), 0.4 mM-NADP⁺, 2 mM-6-phosphogluconate. NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) was assayed in 50 mM-HEPES (pH 7.5), 2 mM-MnSO₄, 0.2 mM-NADP⁺, 2.5 mM-DL-isocitrate. NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41) was assayed in 50 mM-HEPES (pH 7.5), 2 mM-MnSO₄, 0.2 mM-NAD⁺, 2.5 mM-DL-isocitrate. All assays were performed at 25 °C. One unit (U) of activity is expressed as 1 µmol NAD(P)H formed or oxidized min⁻¹.

The protein content and the activities of other enzymes were measured as described by Hondmann *et al.* (1991).

G3P determination. Small portions of frozen mycelium, to which 0.5 ml double-distilled water and 50 µl 60% (w/v) perchloric acid were added, were ground up using a Elvejem tube. The suspension was incubated for 1 h on ice and then centrifuged (10 min, 14000 r.p.m., 4 °C). The supernatant was neutralized by adding 8 M-KOH. G3P content was determined in an enzymic assay containing 1 ml 100 mM-glycine/500 mM-hydrazine (pH 9.6), 0.2 mM-NAD⁺, 1 U G3P dehydrogenase (rabbit muscle) and 50 µl neutralized supernatant. G3P content is expressed in nmol (g dry wt mycelium)⁻¹.

Results

Isolation and growth characteristics of *glcB* pseudorevertants

After mutagenesis the conidiospore suspension was plated on 50 minimal medium agar Petri dishes containing D-glucose/glycerol (50 mM/100 mM) as carbon source. After incubation for 2 d at 37 °C, 1400 growing colonies were found. From these, 500 colonies were tested on a range of media. On glycerol and DHA testplates no colonies were found that grew as well as wild-type WG096, so we concluded that no *glcB*⁺ revertants were generated. It must be pointed out also that growth characteristics of the pseudorevertants selected tended to drift somewhat in successive tests, but we never observed wild-type growth on glycerol or DHA testplates. Of the pseudorevertants isolated, 20 were taken for further analysis.

Genetic characterization of pseudorevertants

The 20 pseudorevertants selected were used to form diploids with tester strain WG202, which has a marker on each chromosome. Diploids derived from each

Table 1. Growth characteristics of *A. nidulans* pseudorevertants outcrossed from *glcB33*

Growth tests were performed at 37 °C as described in Methods. + + + +, Good growth; + + +, moderate growth; + +, poor growth; +, very poor growth; -, no growth. The number of mutations at a specific locus and the chromosome involved are also indicated.

Locus . . .	<i>glcA</i>	<i>glcC</i>	<i>glcH</i>	<i>glcI</i>	<i>glcK</i>	<i>glcL</i>	WG096
Carbon source							
Glucose	++++	++++	++++	++++	+++	+++	++++
Fructose	++++	++++	++++	++++	+++	+++	++++
Acetate	+++	+++	+++	+++	++	++	+++
Glycerol	+	+	+	+++	+	-	+++
DHA	-	+++	+	+	+	-	+++
D-Galacturonate	+	+++	+	+++	+	-	+++
D-Glucose/glycerol	++++	++++	++++	++++	+++	+++	++++
D-Glucose/DHA	++++	++++	++++	++++	+++	+++	++++
No. of pseudorevertants found	12	3	2	1	1	1	
Chromosome involved	V	VI	V	III	II	V	

pseudorevertant and WG202 were haploidized to determine on which chromosome the new mutation was located and to separate it from the *glcB* mutation, provided that it is not located on chromosome I which contains *glcB*. All the mutations could in this way be assigned to a particular chromosome. The outcrossed revertants were then phenotypically characterized. Table 1 lists the phenotype and genotype of the 20 outcrossed pseudorevertants and the number of pseudorevertants found to correspond to various loci.

Three pseudorevertants resembled the *glcC2* mutant, both in linkage group allocation (chromosome VI) and in phenotype (Visser *et al.*, 1988). Complementation analysis with the *glcC2* mutant and with the *glcE1* and *glcF1* mutants (Hondmann *et al.*, 1991), which are also on chromosome VI, revealed that these three pseudorevertants were mutated at the *glcC* locus.

Fifteen pseudorevertants had a mutation on chromosome V. The phenotype of 12 of these pseudorevertants was similar to that of the *glcA1* mutant, which is also on chromosome V (Payton, 1978). These 12 mutants failed to complement *glcA1* in both heterokaryons and diploids. The 12 pseudorevertants did complement non-allelic glycerol non-utilizing mutations in *glcB*, *C*, *D*, *E*, *F* and *G*. These 12 pseudorevertants are therefore considered to be mutated at the *glcA* locus.

Of the three other pseudorevertants with a mutation on chromosome V, two show phenotypical similarities to each other. The outcrossed pseudorevertants grew poorly on glycerol, DHA and D-galacturonate. This might be explained by leaky glycerol kinase mutations, as the glycerol kinase gene is also on chromosome V. However, the two outcrossed pseudorevertants complemented *glcA1* in heterokaryons as well as in diploids. Complementation with the other glycerol non-utilizing mutants revealed that these two pseudorevertants correspond to a new locus. Meiotic analysis of crosses of these two

mutants showed very tight linkage, suggesting they are allelic. The mutants were designated *glcH1* and *glcH2*. Diploids of *glcH1* or *glcH2* and the wild-type grew normally on glycerol, DHA and D-galacturonate, indicating that both mutations are recessive.

The fifteenth pseudorevertant with a mutation on chromosome V grew poorly on a wide range of carbon sources (over 40 different ones were tested), both in a *glcB33* background as well as outcrossed from *glcB33*. The outcrossed mutant and the mutant in a *glcB33* background did not grow on glycerol, DHA or D-galacturonate. Complementation analysis with other glycerol non-utilizing mutants showed that this mutant does not correspond to any of the known glycerol mutations. This mutant was designated *glcL1*.

One pseudorevertant with a mutation on chromosome III was found. When outcrossed from *glcB33*, the mutant grew like the wild-type WG096 on all carbon sources, except on DHA at 37 °C. This mutant complemented all glycerol non-utilizing mutants isolated thus far in *A. nidulans*. This new mutant was designated *glcI1*.

Pseudorevertant number 20 has a mutation on chromosome II. Its phenotypical characteristics resembled that of the *glcL1* mutant, except that the outcrossed mutant did grow on glycerol, DHA and D-galacturonate, albeit much more slowly than the wild-type WG096. The mutant complemented all other glycerol non-utilizing mutants and was designated *glcK1*.

Localization of the *glcA*, *glcH* and *glcL* genes on chromosome V

Analysis of crosses of *glcA1*, *glcH1*, *glcH2* and *glcL1* strains with strains carrying different markers on chromosome V (*nicA*, *pdhB*, *hxA*, *acuG* and *riboD*) showed that the *glcA* and *glcH* genes are located near the *acuG* locus. The *glcL* locus was found to be located

Fig. 1. Map of linkage group V of *A. nidulans*. Map distances are expressed in centiMorgans. For reported marker distances and marker nomenclature see Clutterbuck (1984).

between the *nicA* and *pdhB* locus. A genetic map based on a number of crosses is shown in Fig. 1. Close proximity of *glcA* and *glcH* was confirmed by analysis of crosses of WG196 (*glcA1*) with *glcH1* or *glcH2* strains. The distance between the two genes was estimated to be approximately 3 centiMorgan.

G3P accumulation

Since in strains carrying the *glcB33* mutation no growth is found on media containing mixed substrates like D-glucose and glycerol or D-glucose and DHA, presumably due to G3P accumulation, G3P levels were determined after transfer of mycelium to such media. The levels of G3P found are listed in Table 2.

Pseudorevertants lacking glycerol kinase would not be expected to accumulate G3P on media containing D-glucose/glycerol or D-glucose/DHA, and such accumulation did not occur in the *glcA1* mutant. Low levels of G3P were found in the 12 *glcA glcB33* mutants, of which a typical example is strain *glcA10 glcB33*.

A mutation inactivating the glycerol uptake system, as in the *glcC2* mutant, would, in the presence of *glcB33*, not affect accumulation of G3P on media containing D-glucose/DHA, but would prevent such accumulation on D-glucose/glycerol. This was indeed observed in the three *glcC glcB33* mutants. Data obtained with *glcC10 glcB33* are given in Table 2 as an example.

Both *glcH1 glcB33* and *glcH2 glcB33* show low amounts of G3P on D-glucose/glycerol. The levels found are comparable to those found in the glycerol kinase mutant (*glcA1*). G3P levels in the *glcI1 glcB33*, and to a lesser extent those found in the *glcL1 glcB33* mutant, are higher as in the wild-type WG096, but not as high as in the *glcB33* strain. In the *glcK1 glcB33* strain G3P levels are lower than those found in wild-type WG096, but not as low as those found in the *glcA1* strain.

Glycerol kinase and mitochondrial G3P dehydrogenase activities in the *glcH* mutants

glcH1 and *glcH2* strains were grown for 16 h at 37 °C in minimal medium containing 50 mM-fructose, harvested by filtration and transferred for 6 h to fresh minimal medium containing 100 mM-glycerol or 100 mM-DHA. Glycerol kinase and the flavin-dependent G3P dehydrogenase activities were measured. The activities found are

Table 2. G3P accumulation in glycerol mutants and in pseudorevertants

Mycelia were grown for 16 h on minimal medium with fructose and then transferred for 8 h to fresh minimal medium containing 25 mM-D-glucose/100 mM-glycerol or 25 mM-D-glucose/100 mM-DHA. G3P determinations were done as described in Methods. Values given are means of two determinations. ND, Not determined.

Strain	G3P accumulation [nmol (g dry wt mycelium) ⁻¹]	
	D-Glucose/glycerol	D-Glucose/DHA
WG096	14	20
<i>glcA1</i>	4	3
<i>glcB33</i>	51	90
<i>glcA10 glcB33</i>	5	8
<i>glcC10 glcB33</i>	3	88
<i>glcH1 glcB33</i>	1	ND
<i>glcH2 glcB33</i>	6	ND
<i>glcI1 glcB33</i>	37	51
<i>glcK1 glcB33</i>	7	15
<i>glcL1 glcB33</i>	16	33

Table 3. Glycerol kinase and G3P dehydrogenase (FAD-dependent) activities in *A. nidulans glcH* mutants

Mycelia were grown for 16 h in minimal medium with fructose as carbon source and then transferred for 8 h to fresh minimal medium containing 100 mM-glycerol, 100 mM-DHA or 50 mM-D-fructose. Enzyme activities were determined as described in Methods. Data for WG096 were reported previously (Arst *et al.*, 1990) but are included here to enable comparisons to be made.

	Glycerol kinase [nmol min ⁻¹ (mg protein) ⁻¹]	G3P dehydrogenase (FAD) [nmol min ⁻¹ (mg protein) ⁻¹]
<i>glcH1</i>		
Transfer to D-fructose	≤ 2.0	11
Transfer to glycerol	≤ 2.0	20
Transfer to DHA	≤ 2.0	16
<i>glcH2</i>		
Transfer to D-fructose	≤ 2.0	9
Transfer to glycerol	≤ 2.0	5
Transfer to DHA	≤ 2.0	12
WG096		
Transfer to D-fructose	≤ 2.0	10
Transfer to glycerol	41	53
Transfer to DHA	36	45

shown in Table 3. In both of the *glcH* mutants glycerol kinase and G3P dehydrogenase activities are at uninduced or just slightly elevated levels upon transfer to inducing media containing glycerol or DHA.

Temperature sensitive behaviour of the *glcII* mutant

The *glcII* mutant grew on all carbon sources tested equally as well as the wild-type, except for DHA at 37 °C. A lag time of 3 to 5 d was observed before growth started on DHA at 37 °C. Once started, no growth differences compared to the wild-type were found. However, at lower temperatures (20–30 °C) no lag time was found.

When mycelia of *glcII*, grown in minimal medium containing D-fructose as carbon source at either 30 or 37 °C, were transferred to fresh minimal medium containing 100 mM-DHA at 37 °C, no growth was observed. If transferred to 100 mM-DHA at 30 °C normal growth occurred. Mycelia of *glcII* grown at 30 °C with 100 mM-DHA as carbon source continued to grow when transferred to 37 °C. These transfer experiments indicate that in the *glcII* mutant a factor that is necessary for growth on DHA has become temperature sensitive. Once formed at permissive temperature this factor remains active when mycelium is transferred to a non-permissive temperature. This factor is not formed on D-fructose, but requires DHA to be present. In other transfer experiments with *glcII* it has been found that glycerol, but not D-galacturonate, can also induce this factor at permissive temperatures.

A good correlation was found between the induction of glycerol kinase and FAD-dependent G3P dehydrogenase activities and growth of *glcII*. In transfer experiments allowing growth of *glcII* on DHA, both enzymes were found at induced levels whereas in non-growing mycelia glycerol kinase could not be detected and FAD-dependent G3P dehydrogenase activities were lower than 10 mU (mg protein)⁻¹.

Effect of oxygen tension on the *glcI* phenotype

Under microaerophilic conditions (see Methods) the *glcI* mutant did grow, albeit more slowly than the wild-type, on DHA at 37 °C. On solid media containing glycerol as carbon source growth of *glcI* was also slower than wild-type WG096 under these conditions. An oxygen concentration effect is also observed with *glcA* mutant strains, which did not grow on DHA or glycerol under aerobic conditions. Under microaerophilic conditions, however, the *glcA* mutant grew like the wild-type on solid media containing DHA as carbon source, but it still did not grow on glycerol.

Elevated levels of enzymes at the fructose 6-phosphate level in the *glcL1* mutant

Since the *glcL1* mutant grew slowly on all carbon sources tested, enzyme levels in mycelia grown in minimal medium containing glucose (50 mM) or acetate (100 mM)

Fig. 2. Intracellular phosphatase activities in *A. nidulans* wild-type (Δ) and the *glcK1* mutant (+) grown on minimal medium containing 50 mM-glucose as carbon source. Activities are expressed in nmol *p*-nitrophenol liberated (mg protein)⁻¹ min⁻¹ at 30 °C. A 10 mM-piperazine/glycylglycine buffer was used at all pH values.

as carbon source were compared to the activities found in wild-type WG096. Of all enzymes measured (glycolytic, gluconeogenic, pentose phosphate pathway and some enzymes involved in glycerol and mannitol metabolism) only two activities were found to be significantly altered in the *glcL1* mutant grown on glucose, viz. phosphofructokinase and fructose-6-phosphate reductase. Other enzymes stayed within 40% deviation from wild-type levels. The phosphofructokinase level was 4.3-fold higher than in the wild-type (3125 mU mg⁻¹ vs. 734 mU mg⁻¹), the fructose-6-phosphate reductase activity was 6.1 fold higher (4316 mU mg⁻¹ vs. 707 mU mg⁻¹). Grown on acetate, phosphofructokinase activities were 3.4-fold higher in the *glcL1* mutant (644 mU mg⁻¹ vs. 192 mU mg⁻¹), fructose-6-phosphate reductase activities 2.2-fold higher (828 mU mg⁻¹ vs. 383 mU mg⁻¹) and pyruvate kinase activities 2.1-fold higher (276 mU mg⁻¹ vs. 130 mU mg⁻¹).

Higher intracellular phosphatase activities are found in the *glcK1* mutant

Meiotic crosses of *glcK1* strains with tester strain WG362 indicated that the *glcK* locus is near to the *palcA* locus on chromosome II. We addressed the possibility that the *glcK1* mutant, like *palcA* mutants (Caddick *et al.*, 1986), has an altered phosphatase regulation. Intracellular phosphatase activities in *glcK1* and wild-type mycelium, grown on minimal medium containing glucose as a carbon source, were determined at different pH values. The phosphatase spectra found are shown in Fig. 2. Both acid and alkaline phosphatase activities were approximately 2-fold higher in the *glcK1* mutant compared to the wild-type WG096.

Discussion

The results presented in this paper indicate that the *glcB33* strain is suitable to select pseudorevertants, some of which clearly affect regulation of glycerol metabolism. Since the parental strain has a mutation that leads to G3P accumulation on a mixture of D-glucose and glycerol, pseudorevertants are expected which no longer accumulate G3P or which are less sensitive to G3P accumulation. Two classes of predicted pseudorevertants, following the model for glycerol catabolism proposed by Hondmann *et al.* (1991), are glycerol uptake (*glcC*) mutants and glycerol kinase (*glcA*) mutants. Of the 20 pseudorevertants analysed, twelve were found to be *glcA* mutants and three were found to be *glcC* mutants. This confirms that in *A. nidulans* the main route for glycerol catabolism involves an uptake system, encoded by *glcC*, and substrate phosphorylation by glycerol kinase, encoded by *glcA*.

Of greatest interest, however, were the two *glcH* mutants obtained. In these mutants the enzymes of the aerobic catabolic glycerol pathway are not or are only slightly induced. The low levels of G3P found in these mutants after growth on D-glucose/glycerol are in good agreement with lack of induction. The *glcH* locus is located very close to the *glcA* locus on chromosome V. It seems reasonable to speculate that the *glcH* gene could encode a positive regulator required for pathway-specific induction of glycerol catabolism. For several other catabolic pathways in *A. nidulans* it has been established that a gene encoding a positive regulator is clustered with one or more structural genes controlled by it. Well-known examples of this are the linked *alcR* (positive regulator) and *alcA* (alcohol dehydrogenase I) genes involved in ethanol catabolism (Lockington *et al.*, 1985; Felenbok *et al.*, 1988), the genes involved in the utilization of quinic acid, including probably two regulatory genes (Hawkins *et al.*, 1982; Grant *et al.*, 1988) and the genes, including a regulatory gene, necessary for proline utilization (Arst & MacDonald, 1978; Sharma & Arst, 1985; Hull *et al.*, 1989). An alternative explanation that accounts for the *glcH* characteristics is that the *glcH* gene encodes an enzyme which is involved in the synthesis of the co-inducer for the glycerol catabolic genes. Less likely is that the *glcH1* and *glcH2* mutations are in fact mutations in regulatory regions of the glycerol kinase gene, which not only lead to an uninducible glycerol kinase gene, but also impair full induction of one or more of the genes encoding the FAD-dependent G3P dehydrogenase subunits. In the L-proline catabolism gene cluster of *A. nidulans*, 1 centiMorgan corresponds to a physical distance of 3–4 kb (Hull *et al.*, 1989). If comparable, the distance between *glcA* and the

glcH locus would be in the order of 10 kb, thus making this last explanation unlikely to be true.

The accumulation of G3P on glucose/glycerol and glucose/DHA in a *glcB33* background and enzymic data indicate that in the *glcI1* mutant, under aerobic conditions, the uptake systems for glycerol and DHA, and also DHA reductase and glycerol kinase are functioning properly. Under microaerophilic conditions the *glcI1* mutant does grow on DHA at 37 °C, presumably because the proposed bypass via DHA kinase (Hondmann *et al.*, 1991) can function in the *glcI1* mutant. Why the *glcI1* mutant shows a lag-period of several days before growth on DHA at 37 °C under aerobic conditions starts, remains an unanswered question. Apparently, the *glcI* gene encodes a factor that is essential for aerobic growth on DHA. This factor must also be involved in aerobic glycerol catabolism, otherwise a *glcI1 glcB33* strain would not have been found in the selection scheme used. The nature of the selection makes it unlikely that *glcI* is a component of a DHA detoxification system.

The *glcK1* mutant has lower growth rates, higher intracellular phosphatase activities and in a *glcB33* background low G3P levels. This seems to indicate that the accumulation of G3P in the *glcB33 glcK1* strain can be overcome, at least to some extent, although at cost of loss of ATP. In the outcrossed *glcK1* strain higher levels of intracellular phosphatase activities can account for slower growth rates due to futile cycling of phosphorylated metabolites. Whether the *glcK1* mutant is, in fact, a *palcA* mutant, or only maps near to the *palcA* locus has not been investigated.

The exact nature of the *glcL1* mutation remains to be elucidated. A number of speculations can be made. The *glcL* gene could encode a factor which is essential for the expression of the glycerol kinase gene, and which is also involved in the expression of one or more additional genes. A deficiency of this factor causes slow growth on a wide range of carbon sources and no growth on glycerol, DHA and D-galacturonate. Another possibility is that the *glcL1* mutation represents a mutation in a gene of which the gene-product becomes less sensitive to G3P accumulation. G3P accumulation in the *glcL1 glcB33* mutant is not as high as in the *glcB33* strain, but this difference might be a consequence of slower metabolism.

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