D-GALACTOSE REQUIRING MUTANTS IN *ASPERGILLUS NIDULANS* LACKING PHOSPHOGLUCOMUTASE

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1. Introduction

Sugar residues are essential components of the cell walls and membranes of microorganisms and it is a common observation that mutant strains with enzyme deficiencies in the biosynthesis of specific sugars require that sugar for growth. The cell walls of *Aspergillus* [1] contain predominantly chitin (β 1 → 4 *N*-acetyl D-glucosamine polymer) and glucans (β 1 → 3 D-glucose polymer), while in *A. nidulans* significant amounts of D-galactose and D mannose residues and small amounts of D-arabinose have also been found [2–4]. Temperature-sensitive mutants unable to grow on glucose have been reported which are deficient in chitin synthesis and are repaired by glucosamine [5], and also other mutants requiring mannose at the restrictive temperature [6].

We have predicted that further classes of sugar-requiring mutants are expected [7] and report here the discovery of D-galactose requiring mutants which lack phosphoglucomutase activity.

2. Methods

The strains of *A. nidulans* used were R21 (*pabaA1 yA2*) and R153 (*wA3; pyroA4*). Media were those described in [8].

To grow mycelium for enzyme assays, cultures were inoculated with suspensions of conidia to give 10^6 conidia/ml medium and incubated with vigorous shaking at 37°C. Mycelium was harvested by filtration, washed with distilled water and resuspended in 50 mM potassium phosphate buffer (pH 7.5). Cell-free extracts were prepared by sonication (150W MSE Ultrasonic Disintegrator, peak-to-peak amplitude 12 μM) at 0°C for 1 min and centrifuged at 4°C and 12 000 × *g* for 15 min to remove debris. Protein content was measured with the Folin reagent.

UDP galactose epimerase was assayed by the 2-step procedure [9]:

1. Cell free extract was incubated at 30°C in 50 mM glycine buffer (pH 10.0) with 50 mM UDP galactose in the assay tube. The reaction was stopped after 15 min by heating to 90°C for 30 s, then UDP galactose added to the control tube. Denatured protein was removed by centrifugation.
2. The UDP glucose content was measured with UDP glucose dehydrogenase in a Unicam SP1800 recording spectrophotometer following NADH formation by A_{340} increase. The cuvette contained in 1 ml, sample from step 1, 0.2 M glycine buffer (pH 8.7), 200 units UDP glucose dehydrogenase (from Sigma) and 1 mM NAD.

UDP glucose pyrophosphorylase and phosphoglucomutase were measured in a coupled assay with glucose-6-phosphate dehydrogenase [10]. The formation of glucose-6-phosphate was measured by NADPH A_{340} in a Unicam SP1800 recording spectrophotometer. The reaction mixture contained in 1 ml, 50 mM Tris buffer (pH 8.0), 2 mM $MgCl_2$, 3 units glucose-6-phosphate dehydrogenase (from Sigma), 0.5 mM NADP, 0.01 mM glucose-1:6-bisphosphate and 5 mM pyrophosphate. The overall reaction was started by addition of 5 mM UDP glucose to the sample cuvette. If only phosphoglucomutase was measured, pyrophos-

phate was omitted from the reaction mixture and 5 mM glucose-1-phosphate added to the sample cuvette.

3. Results

3.1. *Isolation of galactose requiring mutants*

Strains requiring D-galactose for growth were isolated in a search for mutants lacking enzymes common to glycolysis and gluconeogenesis. Such mutants are expected to require for growth both a glycolytic carbon source, such as glucose, and a gluconeogenic carbon source, such as acetate, and not to grow when either type of compound is supplied alone. The choice of the two carbon sources presents a problem since the utilisation of one may interfere with the utilisation of the other. We were therefore testing a variety of mixtures amongst which was acetate, a good carbon source for *A. nidulans*, and D-galactose a rather poor carbon source which is partly excluded from the mycelium by acetate [11] and is not a strong carbon catabolite repressor [12].

Suspensions of conidia exposed to ultraviolet irradiation were enriched for glycolytic mutants by incubation in liquid medium with glucose as sole carbon source and periodic filtration to remove mycelium produced by growing conidia. When no further growth was observed (~60 h incubation) the surviving conidia were plated in a minimal medium with galactose (20 mM) and acetate (100 mM). The resulting colonies were screened for glucose non-utilising mutants by replica plating and the mutants then further examined for carbon source utilisation. One group of 4 mutants was found which only grew if D-galactose was present in the medium together with another carbon source. These mutants did not grow on any single carbon source including D-galactose (20 mM), but grew well if galactose (20 mM) was combined with acetate (100 mM) though conidiation was very poor.

The galactose requiring mutants proved identical in further growth tests. Growth and conidiation was very good when galactose (20 mM) was combined with glucose (5 mM), fructose (20 mM), xylose (40 mM) or glycerol (40 mM) and good with ethanol (2.5%, v/v) after a 36 h lag. Poor growth and conidiation occurred on galactose with L-glutamate (40 mM) or L-alanine (50 mM). The galactose requirement was partially met by galactosamine (20 mM), though not by glucosamine (20 mM), in mixtures with acetate, glucose, fructose or glycerol. Lactose (12 mM) supplied as sole carbon source, supported good growth and conidiation, but did not support growth when combined with acetate (100 mM) reflecting the marked sensitivity of β-galactosidase formation to carbon catabolite repression in *A. nidulans* [13].

In all conditions of growth the morphology of hyphae and colonies was normal, though the amount of conidiation was markedly diminished on mixtures of galactose with gluconeogenic carbon sources compared to mixtures with other glycolytic carbon sources. When incubated on solid media without galactose conidia germinated yielding highly branched compact clusters of mycelium which ceased growth but was not swollen or distorted.

Since galactose non-utilising mutants in *A. nidulans* lacking galactokinase or galactose-1-phosphate uridyl transferase grow well on glucose and other carbon sources [14] it seemed most likely that these new mutants are deficient in the biosynthesis of D-galactose residues and lack either UDP galactose-4-epimerase, UDP glucose pyrophosphorylase or phosphoglucomutase.

3.2. *Enzyme assays with the galactose requiring mutants*

None of the mutants showed a marked deficiency in UDP galactose epimerase activity (table 1). However when assayed for the overall activity of UDP glucose pyrophosphorylase together with phosphoglucomutase, by supplying UDP glucose as substrate and measuring the glucose-6-phosphate formed with glucose-6-phosphate dehydrogenase, each of the mutants showed virtual absence of overall activity. Assaying for phosphoglucomutase alone by supplying glucose-1-phosphate substrate showed that each of the mutants lacks this activity (table 1). This was confirmed since overall activity in the assay system with UDP glucose substrate was restored when phosphoglucomutase (Sigma) was added to the mutant extracts.

We therefore conclude that the mutants lack phosphoglucomutase and designate them *pgm*.

3.3. *Properties of the* pgm *mutants*

Heterozygous diploid strains *pgm*1/+ and *pgm*3/+

Table 1
Enzyme activities in galactose requiring mutants

Strain	Enzyme spec. act. (mM product $.h^{-1}.mg^{-1}$)	
	UDP Galactose epimerase	Phosphogluco-mutase
R21 (wild-type)	91	78
*pgm*1	75	0.1
*pgm*2	44	11
*pgm*3	73	0
*pgm*4	181	0.7

Mycelium was grown for 18 h on defined medium with D-galactose (20 mM) and acetate (100 mM). Cell free extracts were prepared and assayed as in section 2

had no requirement for galactose demonstrating that the *pgm* mutations are recessive. The four *pgm* mutants fail to complement when combined in heterokaryons and therefore identify a single gene *pgm*A which has been located in linkage group VI by mitotic haploidisation [15].

Pleiotropic mutants isolated by resistance to L-sorbose have been described which map at the *sor*B locus in linkage group III and also confer compact colony morphology and 90% deficiency in phosphoglucomutase [16]. However the *pgm*A mutants described here do not affect colony morphology, are fully sensitive to L-sorbose (25 mg $.ml^{-1}$) in the presence of galactose (20 mM) and acetate (100 mM) and are thus clearly distinct from the *sor*B mutants.

4. Discussion

The presence of D-galactose in the cell wall of *A. nidulans* is firmly established by analysis [2–4] and by the direct demonstration of the incorporation of isotopically labelled galactose residues into cell wall polymers in a mutant lacking galactose phosphate-UDP glucose transferase [17]. The phosphoglucomutase *pgm*A mutants have an absolute requirement for galactose and therefore show the essential role of the sugar for cell wall biosynthesis in *A. nidulans*. In a similar fashion a requirement for D-mannose was shown [6] for normal cell wall synthesis in a mannose requiring mutant *mnr*A deficient in phosphomannose mutase. The two sugars each constitute 5–10% dry wt of cell wall material. The properties of the sugar requiring mutants strongly suggest that both galactose and mannose moeities occur in polymers essential for cell wall synthesis and integrity, and not in polymers such as $\alpha\ 1 \rightarrow 3$ glucan which are dispensible for growth [18] and appear endogenous carbohydrate reserves supporting processes occurring after primary growth [19].

The almost complete absence of phosphoglucomutase activity in the single gene *pgm*A mutants of *A. nidulans* indicates a single enzyme species in the organism. The same result is found in *Escherichia coli* where mutants at the *pgm* locus totally lack phosphoglucomutase activity, grow very poorly on galactose and are deficient in the synthesis of UDP glucose and UDP galactose [20]. On the other hand in *Saccharomyces cerevisiae* [21], in *Neurospora crassa* [22] and in *N. sitophila* [23] there are two distinct phosphoglucomutase isoenzymes. In *S. cerevisiae ga*5 mutants retain 20% of the wild-type activity but fail to grow on galactose since they lack the major (catabolic) isoenzyme. However the *ga*5 mutants grow normally on glucose showing that the activity of the minor isoenzyme is sufficient for biosynthesis [21]. In *Neurospora*, mutants with deficiencies in the isoenzymes were found among strains isolated by their abnormal colony morphology on media in which glucose is the principal carbon source. The *rg*I (ragged colony) and *rg*II mutations are in separate structural genes for each of the isoenzymes which interact in a complex fashion [23]. It is striking that the *pgm*A mutants of *A. nidulans* have normal colony morphology when utilisable carbon source is suitably supplemented with galactose. (Reduced density of conidiation in

the *pgm*A mutants most probably reflects deficiency in the elaboration of storage glucans utilised in sporulation [19].) The presence of a single phosphoglucomutase in *A. nidulans* requires proof by study of the enzyme protein and it is possible that the *sor*B mutants [16] represent either a second isoenzyme or a second genetic locus regulating enzyme formation. However the clear genetic and phenotypic distinctions between the *pgm*A and *sor*B mutants suggest to us that the *sor*B locus has an unidentified primary function loss of which generates pleiotropically the secondary characters of phosphoglucomutase deficiency, L-sorbose resistance and abnormal colony morphology.

Clearly other galactose requiring mutants in *A. nidulans* deficient in the biosynthesis of UDP galactose from glucose-6-phosphate can be expected with lesions in UDP glucose pyrophosphorylase or UDP galactose epimerase. It would also be of interest for the study of cell wall biosynthesis to seek mutants requiring other specific carbohydrates, e.g., D-arabinose which has been reported in the cell wall [3].

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