Characterization of *Aspergillus nidulans* Mutants in Carbon Metabolism Isolated after D-Galacturonate Enrichment

By J. H. A. A. UITZETTER,† C. J. BOS AND J. VISSER*

Department of Genetics, Agricultural University, Gen. Foulkesweg 53, 6703 BM Wageningen, The Netherlands

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A selective method for the isolation of *Aspergillus nidulans* mutants defective in the pyruvate dehydrogenase complex was devised. The essential steps in the procedure were a mutagenic treatment of conidia with X-rays to about 50% survival, followed by filtration enrichment in minimal medium with D-galacturonate as sole carbon source, and rescue on complete medium with acetate. The mutants thus isolated were phenotypically characterized on the basis of growth tests, and different genotypes were assigned on the basis of complementation tests. The majority of the mutants that were unable to utilize galacturonate were defective in one of the components of the pyruvate dehydrogenase complex. In addition, mutants defective in pyruvate carboxylase, mutants defective in glycerol catabolism and some novel mutants which were only unable to use D-galacturonate as carbon source were found. At least two genes were shown to be involved in D-galacturonate metabolism.

INTRODUCTION

Aspergillus nidulans mutants defective in carbon metabolism have been found after mutagenic treatment, with a low percentage of survival without further enrichment procedures (Skinner & Armitt, 1972). Carbon metabolism mutants are commonly selected by a filtration enrichment technique. This method has been used to isolate both gluconeogenic (Armitt *et al.*, 1976) and glycolytic mutants. For the isolation of the latter a disaccharide like sucrose (Payton *et al.*, 1977) or a readily metabolized monosaccharide like glucose (Payton *et al.*, 1977; Bos *et al.*, 1981) were used.

We reported previously growth of *A. nidulans* on D-galacturonate as sole carbon and energy source (Uitzetter *et al.*, 1982). Tests with mutants defective in pyruvate kinase (*pki*) and the pyruvate dehydrogenase complex (*pdh*) showed good growth of the former but no growth of the latter mutants on this substrate. This indicates a metabolic pathway for D-galacturonate leading to pyruvate as one of the degradation products. Such a pathway exists in *Escherichia coli* (Ashwell *et al.*, 1958). Payton *et al.* (1977) isolated eight *pdh* mutants among some 200 mutants selected for their inability to grow on sucrose. They used *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a mutagen at a 0.5% survival level.

Genetic analysis of these *pdh* mutants showed that the genes *pdhA* and *pdhB* belong to linkage groups I and V respectively. Bos *et al.* (1981) reported the isolation of a number of other pyruvate dehydrogenase complex mutants which were obtained by UV-treatment at a 75–80% survival rate followed by enrichment in glucose minimal medium. Of the 160 strains isolated, 54% had defective pyruvate dehydrogenase complex activity. Among these mutants a third distinct gene, *pdhC*, was found, and assigned to linkage group VIII. In order to obtain other mutants defective

† Present address: Netherlands Organization for the Advancement of Pure Research (ZWO), The Hague, The Netherlands.

Abbreviations: CM, complete medium; MM, minimal medium.

in pyruvate metabolism, D-galacturonate was included in the medium for enrichment. This paper describes the results of an X-ray mutation experiment with *A. nidulans* followed by D-galacturonate enrichment, and the characterization of mutant classes found.

METHODS

Strains. The *Aspergillus nidulans* strain used for mutation was WG145 (*wA3 pyroA4*). This strain has wild-type characteristics for carbon source utilization. For other experiments the following strains were used: WG001 (*yA2 phenA2 lysB5*); WG203 (*yA2 meaA3 pA1 sB3 nicB8 riboB2*); WG097 (*pabaA1 yA2 pdhA1*); WG100 (*pabaA1 yA2 pdhB1*); WG107 (*pabaA1 yA2 pdhC1*); WG102 (*pabaA1 yA2 pycA2*); WG135 (*pabaA1 yA2 pycB4*); WG196 (*pabaA1 yA2 glcA1*) and WG197 (*pabaA1 yA2 glcB33*). These strains were derived from the original Glasgow strains of *A. nidulans* (Pontecorvo *et al.*, 1953). All symbols have their usual meaning (Clutterbuck, 1973; Payton, 1978). For mutants concerned with D-galacturonate metabolism the gene symbol *gaa* is used.

Media and growth tests. *A. nidulans* WG145 was grown on complete medium (CM) according to Pontecorvo *et al.* (1953) except that glucose was replaced by acetate (0.1 M) + sucrose (0.01 M). Growth tests were done on minimal medium (MM) supplemented with pyridoxine.HCl (1 mg l⁻¹). The medium was solidified with 1.5% (w/v) agar. Unless stated otherwise glucose was omitted and other carbon sources were added instead. Carbon sources (pH 6.0) were added separately as membrane filter sterilized solutions.

The final concentrations of the carbon sources were: D-galacturonate 0.05 M; D-gluconate 0.05 M; D-glucose 0.05 M; glycerol 0.05 M; D-xylose 0.05 M; acetate 0.1 M; acetate 0.1 M + sucrose 0.01 M; L-glutamate 0.05 M + D-fructose 0.01 M; glycerol 0.05 M + acetate 0.1 M. All acids were used as their sodium salts. The growth of the wild-type and mutant strains was scored after 2 d incubation at 37 °C.

Induction and isolation of mutants. Mutations were induced by X-ray treatment for 6.5 min at a dose yielding 51% survival, using a Philips Röntgen apparatus equipped with a MCN 161 tube (160 kV, 19 mA, 2 mm Al-filter) placed 36 cm from the bottom of a Petri dish which contained 15 ml of a conidial suspension of strain WG145 (about 5×10^7 conidia ml⁻¹). The total dose was 2.5 krad. Survival was determined before and after irradiation by plating a diluted suspension of conidia on CM acetate. Irradiated conidia were transferred to 300 ml liquid MM in a 1 litre infusion bottle with D-galacturonate as carbon source. The flask was incubated in a reciprocal shaker bath (160 strokes min⁻¹) at 37 °C for 12 h. Conidia that did not germinate were separated from hyphae by filtration over cotton wool; the conidia were then collected on a membrane filter and incubated in 100 ml fresh medium for a further 11 h. Finally the conidia that had still not germinated and that had passed a second filtration procedure were resuspended in 0.5 ml 0.8% (w/v) NaCl. After appropriate dilution the suspension was plated at a density of about 60 colonies per plate in CM with acetate and incubated at 37 °C for 2 d. From the colonies thus obtained master plates were made on CM with acetate and replicas were made on MM with D-galacturonate and acetate respectively to identify carbon metabolism mutants. Mutants which showed leaky growth or no growth on D-galacturonate were then purified by plating conidia from a single mutant colony. Monospore cultures were made and conidia were preserved on dehydrated silica gel according to Perkins (1962).

Phenotypic classification. To characterize the mutants by their phenotype, growth tests were done as described above. Criteria to assign phenotypes are shown in Table 1.

Complementation tests. To assign mutant genes, *pdhA*, *pdhB* and *pdhC*, and *pycA* *pycB* tester strains with different colour and vitamin markers were used for complementation. Heterokaryotic mycelium was obtained according to Pontecorvo *et al.* (1953). Twin pieces of heterokaryotic mycelium were transferred to MM with sucrose and to MM with acetate + sucrose. The latter medium gave much better growth than acetate alone. Complementation with *glc* mutants was done with diploids constructed with the *glcA1* and *glcB33* strains respectively. Growth tests with diploids were done on MM with glycerol and galacturonate respectively, using MM with sucrose as a control.

Enzyme assays. Uronic acid isomerase (EC 5.3.1.12), D-altronic acid dehydrogenase (EC 1.1.1.58) and D-altronic acid hydrolyase (EC 4.2.1.7) were assayed according to Ashwell (1962).

Chemicals. D-Galacturonic acid was obtained from Aldrich. Tagaturonic acid was synthesized according to Ehrlich & Guttmann (1934); altronic acid was synthesized according to Pratt & Richtmeyer (1955). These acids were a gift from the Department of Organic Chemistry, Agricultural University, Wageningen, The Netherlands. All other chemicals were obtained from Merck.

RESULTS AND DISCUSSION

The survival of the irradiated conidia was 51%. In order to prevent background damage a relatively low dose rate was used in this mutation experiment. The mutagenesis and enrichment procedure resulted in 2232 colonies, of which 840 were tested for their ability to grow on D-galacturonate. From these, 209 (25%) showed little or no growth on this carbon source and were

Table 1. Growth criteria used to assign phenotypes to isolated *A. nidulans* mutants

Carbon source	Phenotype . . .	Growth*				
		Wild-type	Gaa ⁻	Glc ⁻	Pyc ⁻	Pdh ⁻
D-Galacturonate		++++	-/+	-	-	-
Glycerol		+++	+++	-	++	-/+
Glycerol + acetate		+++	+++	-	+++	+++
D-Glucose		++++	++++	++++	-	-
D-Xylose		++++	++++	++++	-	-
D-Gluconate		++++	+++	+++	+	-
L-Glutamate + D-fructose		++++	++++	++++	+++	-
Acetate + sucrose		+++	+++	+++	+++	+++
Acetate		+++	+++	+++	+++	+++
Totals†			15	36	17	132
Frequency (%)			7	17	8	63

* + + + +, good; + + +, moderate; + +, poor; +, very poor; and -, no growth.

† Nine strains could not be assigned with these criteria.

therefore of the desired phenotype; 145 (17%) appeared to have other deficiencies and were not considered further. Table 1 summarizes the criteria which were used to assign different phenotypes to the D-galacturonate non-utilizing or ill-utilizing mutants. Growth behaviour on the various substrates was discussed by Uitzetter *et al.* (1982).

The Pdh⁻, Pyc⁻ and Glc⁻ phenotypes classified by these growth tests were then shown by complementation to correspond with genotypes already known; their frequencies are shown in Table 1. Mutants in glycerol metabolism (*glc*) and D-galacturonate metabolism (*gaa*) showed normal sporulation like the parental strain, in contrast to *pdh* and *pyc* mutants. The number of mutants recovered was very high. This could have been due to a relatively quick growth of the mycelium which made the enrichment period sufficiently short to avoid loss of mutants by cross-feeding and aggregation. Only nine mutants could not be assigned by the phenotypic characterization.

Pyruvate dehydrogenase complex (*pdh*) mutants

The majority (63%) of the isolated mutants turned out to have a defective pyruvate dehydrogenase complex. Complementation of these mutants was done with the three marker genes known for this complex. *pdhA* is the structural gene for lipoate acetyltransferase (E₂), and *pdhC* and *pdhB* are the structural genes for the two subunits of pyruvate decarboxylase (E₁). The distribution of the *pdh* mutants over the three genes was 37% *pdhA*, 28% *pdhB* and 35% *pdhC*. It is obvious that other selection techniques are required to find mutants with defects in structural genes coding for the other complex components, viz. lipoamide dehydrogenase (E₃) and the two regulating enzymes pyruvate dehydrogenase kinase and pyruvate dehydrogenase-phosphatase. The pyruvate dehydrogenase kinase, which is firmly bound to the core of the complex, inactivates the complex by phosphorylating the α subunit of E₁. A specific phosphatase, which is loosely associated with the complex, reactivates the enzyme (Hucho *et al.*, 1972).

A possible reason for missing the E₃ mutants of this complex might be the occurrence of more than one gene coding for E₃. For instance, in mammalian tissues several lipoamide dehydrogenase isoenzymes have been found which are functionally exchangeable between the pyruvate and 2-oxoglutarate dehydrogenase multienzyme complexes (Kenney *et al.*, 1972; Sakurai *et al.*, 1970). Of course, *pdh* kinase mutants will not be found as the complex will be fully active under those circumstances. In the case of *pdh*-phosphatase mutants a partially active complex may still be present since phosphorylation is a post-translational event. This will result in loss of such mutants in an early stage of the enrichment. Also it is not known whether non-specific phosphatase activities present act to some extent on the phosphorylated pyruvate dehydrogenase complex.

Glycerol metabolism (glc) mutants

All the *glc* mutants isolated belonged to a single class as shown by complementation in diploids; they were identified as *glcB*. It was necessary to construct diploids for complementation analysis because heterokaryons showed very poor growth, if any, on glycerol (McCullough *et al.*, 1977). *glc* mutants were first isolated by Payton (1978) after mutagenic treatment followed by enrichment in glycerol medium. He assigned two genes: *glcA* (linkage group V) and *glcB* (linkage group I). The *glcA* mutant showed very poor growth on glycerol. The *glcB* mutant was absolute, and its growth on other carbon sources in the presence of glycerol was strongly inhibited. The exact nature of the *glcA* and *glcB* mutants is still unresolved. The *glcB* mutant was suspected to be deficient in glycerol-3-phosphate dehydrogenase because of the interference of glycerol with growth on other carbon sources, which is also observed in *E. coli* (Cozzarelli *et al.*, 1965) and *Salmonella typhimurium* (Aceves-Pina *et al.*, 1974). We were unable, however, to come to this conclusion since we never detected a glycerol kinase activity in these fungal extracts, which makes it unlikely that *glcB* corresponds to a *sn*-glycerol-3-phosphate dehydrogenase defect. Recently, we have found a NADP⁺-dependent glycerol dehydrogenase activity as well as a weak glyceraldehyde kinase activity in crude extracts of wild-type *A. nidulans* mycelium (J. Visser and co-workers, unpublished results) which suggests another pathway. Both *glcA* and *glcB* failed to grow on D-galacturonate, indicating that glycerol and D-galacturonate catabolism have at least one step in common.

Pyruvate carboxylase (pyc) mutants

Skinner & Armitt (1972) were the first to describe *pyc* mutants after mutagenic treatment of *A. nidulans* conidia to 1% survival. They tested 8000 colonies of which 3 among 55 sucrose-negative survivors had the *pyc* genotype. A large number of *pyc* mutants have since been isolated by Payton *et al.* (1977) and Bos *et al.* (1981). Complementation data suggest intragenic complementation, although the existence of two closely linked genes is not completely excluded (C. J. Bos, unpublished results).

In this experiment 17 mutants showed a Pyc⁻ phenotype (Table 1). They were completely unable to use D-galacturonate for growth, in contrast to the *pyc* mutants isolated earlier (Bos *et al.*, 1981). The *pyc* genotype of these mutants was confirmed by complementation which occurred only with one of the two *pyc* test strains (WG102 and WG135).

D-Galacturonate metabolism (gaa) mutants

In the mutation experiment described, 15 mutants were isolated which were exclusively unable to use D-galacturonate as sole carbon and energy source. These mutants showed normal growth on the other carbon sources listed in Table 1, indicating that D-galacturonate was not catabolized through one of the main pathways for carbohydrate degradation. Apart from the carbon sources already mentioned the following compounds were tested for growth; D-gluconate, D-glucarate, D-glucuronate, D-altronate, D-galactarate and D-tagaturonate. All compounds except the latter two were able to support growth both in wild-type and *gaa* mutants.

In *E. coli* a uronic acid catabolic pathway has been demonstrated (Ashwell *et al.*, 1958). After an initial isomerization reaction, a dehydrogenation leads to D-altronate. The next steps involve dehydration of the last intermediate to 2-keto-3-deoxy-gluconic acid and subsequent phosphorylation. Finally an aldolase reaction leads to pyruvate and glyceraldehyde 3-phosphate as end products. In preliminary experiments with the wild-type strain of *A. nidulans* grown on D-galacturonate none of the reactions mentioned above could be detected, either in cell-free extracts, or as membrane-bound activity. Therefore, another D-galacturonate degradation pathway is likely to exist in this fungus. Alternative routes have been observed in *Pseudomonas* spp. (Kilgore & Starr, 1959; Preiss & Ashwell, 1963*a, b*), and in *Agrobacterium tumefaciens* (Chang & Feingold, 1969), where oxidation of D-galacturonate to D-galactarate is the initial step. The specific pathway in *A. nidulans* is unknown, but from the growth characteristics of *pdh*, *pyc* and *pki* mutants it is clear that pyruvate is one end-product. On the basis of the phenotype of the *glcA* and *glcB* mutants D-glyceraldehyde is suggested to be the intermediate which the two routes have in common. This implies a non-phosphorylative breakdown route of D-galacturonate in this fungus.

Table 2. Allocation of *gaaA* and *gaaB* by mitotic haploidization of diploids *gaaA*/WG203 and *gaaB*/WG203

	Markers in:		No. of haploids			
	Mutant strain	Tester strain	<i>gaaA1</i>	<i>gaaA</i> ⁺	<i>gaaB2</i>	<i>gaaB</i> ⁺
I	<i>yA</i> ⁺		0	0	4	1
		<i>yA2</i>	20	20	0	39
II	<i>wA</i>		12	29	25	90
		<i>wA</i> ⁺	20	20	4	39
III	<i>meA</i> ⁺		0	3	*	*
		<i>meA3</i>	32	46	*	*
IV	<i>pyroA4</i>		15	14	13	57
		<i>pyroA</i>	17	35	16	72
V	<i>pA</i> ⁺		*	*	13	58
		<i>pA1</i>	*	*	16	71
VI	<i>sB</i> ⁺		32	1	16	53
		<i>sB3</i>	0	48	13	76
VII	<i>nicB</i> ⁺		17	25	9	77
		<i>nicB8</i>	15	24	20	52
VIII	<i>riboB</i> ⁺		20	20	13	56
		<i>riboB2</i>	12	29	16	73

* *meA3* and *pA1* were difficult to classify.

Existence and allocation of different *gaa* genes

In order to allocate *gaaA* a heterozygous diploid was constructed with master strain WG203, and haploid segregants were induced with *p*-fluorophenylalanine according to McCully & Forbes (1965).

From the results shown in Table 2 it can be concluded that the *gaaA* gene belongs to linkage group VI, since genes on this chromosome remain together during mitotic haploidization. Because all *gaa* strains isolated were in the *wA3 pyroA4* background one of the haploid segregants was used for complementation experiments with the other *gaa* mutants isolated. Heterokaryons were tested on MM with sucrose and MM with galacturonate respectively. The *gaaA1* strain was able to complement with all other *gaa* mutants, but not with the parental strain from which it was derived.

One of the other *gaa* mutants (*gaaB2*) was also analysed in a heterozygous diploid with the same master strain (Table 2). Because a conclusive assignment of *gaaB* was impossible, a second heterozygous diploid was constructed with WG001 in order to examine the segregation of markers on chromosome I, III and V (results not shown). The results indicated that *gaaB* is located on linkage group I which confirms that at least two genes exist coding for enzymes in D-galacturonate metabolism.

Conclusions

D-Galacturonate is a very suitable substrate for enrichment of mutants defective in galacturonate, glycerol and pyruvate metabolism. The defined classes of mutants thus derived undoubtedly give a tool for resolving the subsequent steps of both D-galacturonate and glycerol metabolism. At least two genes, *gaaA* and *gaaB*, are involved in D-galacturonate breakdown. They are located on linkage groups VI and I respectively. Since *pki* mutants grew on D-galacturonate whereas *pdh* and *pyc* mutants did not, this indicated that galacturonate is degraded to a metabolite beyond phosphoenolpyruvate, viz. pyruvate. In *E. coli* D-galacturonate degradation finally leads to D-glyceraldehyde 3-phosphate and pyruvate as end-products. The isolation of a large number of *glcB* mutants by galacturonate enrichment indicates that in *A. nidulans* glycerol catabolism has at least one step in common with D-galacturonate breakdown. Since dehydrogenation of glycerol seems to be the initial step in the utilization of this substrate by *A. nidulans* it is postulated that D-glyceraldehyde is an intermediate in both pathways. Detailed studies on glycerol metabolism are in progress, but thus far we have not been able to identify which enzyme function is coded by the *glcB* gene.

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