MUTANTS OF *ASPERGILLUS NIDULANS* LACKING PYRUVATE CARBOXYLASE

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1. Introduction

The tricarboxylic acid cycle serves a dual role during the growth of many organisms, it is the terminal respiratory pathway and its intermediates are the precursors for the biosynthesis of many cell constituents. Growth of microorganisms on glucose, glycerol or other substrates catabolised to C_3-acids necessitates anaplerotic [1] carbon dioxide fixation. The substrate for this carboxylation reaction in Enterobacteriaceae is phosphoenol pyruvate (PEP) [2] whereas in fungi such as yeast [3] and *Neurospora crassa* [4] pyruvate is carboxylated by the enzyme pyruvate carboxylase to yield oxaloacetate. Mutants unable to synthesise C_4-intermediates from carbon dioxide and C_3-acids are recognised by their failure to grow on glucose or glycerol unless supplemented with a source of C_4-intermediates such as succinate or glutamate. They can also grow upon acetate since net C_4-synthesis is then effected by the glyoxylate cycle [5]. Such mutants have been reported in *N. crassa* [6, 4] and in *Escherichia coli* [7, 9]. In *E. coli*, they were the origin of secondary mutants, which still lacked PEP carboxylase, but now grew upon C_3-acids alone since they had become constitutive for the enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase [7]. We have sought to isolate pyruvate carboxylase mutants in *Aspergillus nidulans* in order that we might, by similar procedures, derive mutants of this organism constitutive for enzymes of the glyoxylate cycle.

This letter describes the isolation and properties of mutants of *A. nidulans* lacking pyruvate carboxylase activity. The 3 mutants recovered identify a single gene designated *pyc* and located in linkage group III.

2. Experimental procedures

Routine techniques for genetic analysis in *A. nidulans* were employed [9, 10]. Cultures for the determination of pyruvate carboxylase activity were grown in a defined mineral salts medium [10] to which $MgSO_4$ and carbon sources were added separately after sterilization, sodium acetate and sodium glutamate having first been brought to pH 6.5. Cultures were started by inoculation with suspensions of washed conidia yielding 10^6 conidia/ml of medium and incubated at 37° with vigorous shaking. Mycelia were harvested by filtration, washed with distilled water and resuspended in buffer at pH 7.5 containing 20 mM Tris-HCl, 2 mM $MgCl_2$ and 1 mM EDTA (disodium salt). Cell free extracts for enzyme assay were prepared by sonicating the mycelia at 0–4°. Protein concentrations were measured as previously described [11].

Pyruvate carboxylase activity was measured by the method described by Sundaram et al. [12], in which the amount of ^{14}C-oxaloacetate formed from pyruvate and ^{14}C-bicarbonate in 10 min at 30° was determined as ^{14}C-malate in the presence of malate dehydrogenase and excess NADH.

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3. Results and discussion

3.1. *Pyruvate carboxylase activity in mycelia grown upon different carbon sources*

Pyruvate carboxylase in mycelia grown on sucrose, glucose or glycerol, when the enzyme is necessary for anaplerotic C_4-synthesis, was found at similar specific activities though somewhat greater amounts were consistently present in sucrose grown organisms. After growth on acetate, when anaplerotic synthesis of C_4-intermediates is carried out by the enzymes of the glyoxylate cycle [11, 13], pyruvate carboxylase was again found at the same specific activity as in mycelia grown on glycolytic carbon sources (table 1). It thus appears that pyruvate carboxylase is synthesized constitutively in *A. nidulans* and not subject to coarse regulation.

When pyruvate was omitted from the enzyme assay mixture there was no incorporation of isotope into acid stable material. Omission of acetyl co-enzyme A caused little loss of activity, but since this compound may be present at significant concentrations in crude extracts it is not possible to deduce if acetyl-CoA is required for activation of the enzyme in *A. nidulans* as it is in most other organisms [14].

Table 1
Pyruvate carboxylase activity in mycelia grown on different carbon sources.

Carbon source	Conc. (M)	Period mycelia grown (hr)	Specific activities*		
			Experiment 1	Experiment 2	Experiment 3
Sucrose	0.02	18	2.92	3.83	2.19
Glucose	0.02	18	1.83	1.27	0.80
Glycerol	0.04	21	1.84	1.30	1.76
Acetate	0.10	24	2.61	2.34	1.45

* Pyruvate carboxylase activity was measured by formation of ^{14}C-oxaloacetate from ^{14}C-bicarbonate and pyruvate in the presence of malic dehydrogenase and NADH [12]. Specific activities are given as μmoles ^{14}C-bicabonate fixed/mg protein/hr.

3.2. *Mutants lacking pyruvate carboxylase activity*

Mutants unable to grow on sucrose were isolated after mutagenesis of conidia of strain R21 *paba 1 y* (requires *p*-amino-benzoic acid; yellow conidia) with *N*-methyl-*N*-nitroso-*N'*nitroguanidine using the procedure of Alderson and Hartley [15]. The surviving conidia (about 1%) were plated on minimal medium with acetate as carbon source to give about 20 colonies per plate, and these were replicated onto sucrose minimal plates. Altogether 55 mutants not growing on sucrose were found among approximately 8000 colonies tested. The mutants were then tested for growth on glycerol (0.04 M), glutamate (0.02 M) and (0.02 M sucrose + 0.04 M glutamate). Many of the mutants grew on glutamate alone but failed to grow on (sucrose + glutamate), indicating that the metabolic lesions in these organisms caused accumulation of inhibitory intermediates from sucrose. However 4 mutants showed some growth on (sucrose + glutamate), the phenotype predicted for a pyruvate carboxylase mutant, although growth was much less vigorous than expected. The 4 mutants were tested for enzyme activity after growth with acetate as carbon source, and 3 found defective: no pyruvate carboxylase activity was detected in 2 of the mutants (*pyc2* and *pyc3*) while the third (*pyc1*) had about 10% of wild type activity (table 2).

Each of the pyruvate carboxylase mutants segregates in crosses as a single gene mutation. All 3 revert readily and thus are apparently not deletions. Tests in heterokaryons show that *pyc1* and *pyc2* do not complement *pyc3* and that the 3 mutants are therefore functionally allelic. (Growth of the heterokaryon between *pyc1* and *pyc2* when tested on sucrose-containing medium is interpreted as the result of interallelic complementation). Accordingly, the 3 mutants have been allocated to a single gene, called *pyc*, and most likely the structural gene for pyruvate carboxylase. It maps in linkage group III close to the methionine marker *meth2* (about 5% recombination).

3.3. *Response of the pyruvate carboxylase mutants to different carbon sources*

It was noted during the analysis of crosses between the *pyc* mutants and strains having markers for nutritional requirements or sugar utilisation, that although the pyc recombinants failed to grow on sucrose plates, they grew well on plates containing galactose as carbon source together with several amino acids. The *pyc* mutants were therefore tested for growth on different carbon sources supplemented with various amino acids which could supply C_4-intermediates. It was found

Table 2
Mutants of *A. nidulans* lacking pyruvate carboxylase activity.

Strain	Specific activities*		
	Experiment		
	1	2	3
R21 *pyc*$^+$	1.33	1.44	1.06
R21 *pyc1*	0.15	0.14	0.08
R21 *pyc2*	<0.01	<0.01	<0.01
R21 *pyc3*	<0.01	<0.01	<0.01

* See table 1.
Organisms were grown with acetate as carbon source.

that growth on sucrose supplemented with either glutamate, proline or arginine was very poor. Glucose supplemented as above gave better growth; and the growth response to lactose, galactose, fructose or glycerol with these amino acids was good. In all cases proline proved a better supplement than arginine or glutamate. The poor response of the *pyc* mutants to (sucrose + glutamate) makes clear the reason for our difficulty in isolating the mutants since this combination had been the basis of earlier screening programmes. Differences in growth response to the different glycolytic carbon sources supplemented with glutamate are not understood. However it is notable that 3 of the better carbon sources for the *pyc* mutants, galactose [10], lactose [16, 17] and glycerol are utilised for growth after an adaptive lag.

3.4. *The anaplerotic function of pyruvate carboxylase*

The isolation of mutants lacking pyruvate carboxylase activity in *A. nidulans* and their absolute requirement for a supply of C_4-intermediates in growth on glycolytic carbon sources, provides good evidence for the physiological function of this enzyme in the organism. The observations reported in this paper confirm those made in other organisms and support the deduction of the essential anaplerotic function of pyruvate carboxylase [1].

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