Agar as a Carbon Source and its Effect on the Utilization of Other Carbon Sources by Acetate Non-utilizing (*acu*) Mutants of *Aspergillus nidulans*

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INTRODUCTION

The presence of two major types of polysaccharide in agar – neutral agarose and charged agaropectin – was first recognized by Araki (reviewed 1959). More recently agar has been shown to consist of a family of linear polymers of variously modified galactose residues (Duckworth & Yaphe, 1971). The best characterized of these polymers is agarose, the principal gel-forming agent in agar, in which 3-linked β -D-galactopyranose residues and 4-linked 3,6-anhydro- α -L-galactopyranose residues are arranged alternately (Rees, 1969). This polymer probably forms a network gel through crosslinking of helical chains between regions of double-helix (Rees, 1972). The agaropectins are a group of less well defined molecules of the same polymer structure as agarose but with regions in which the galactopyranose units are substituted with sulphate, pyruvic acid acetals or methoxy residues (Rees, 1969; Young, Duckworth & Yaphe, 1971).

The presence in agar of small amounts of compounds promoting microbial growth has been recognized, and these contaminating nutrients, often vitamins, can be readily removed by washing the agar in water (Ryan, 1950). Agar may also contain antagonists to microbial growth. Inhibition of bacterial growth by long-chain fatty acids, which could be removed by washing in alcohol, has been noted (Sykes, 1956). Virus growth may be affected, and in some cases the inhibiting agent, which can be removed by washing the agar in water, appears to be an agaropectin component (Takemoto & Liebhärber, 1961). Agar can also antagonize the activity of some microbial growth inhibitors. Thus the concentrations of phenols (Sands & Bennett, 1964) and of certain antibiotics (Iyer & Iyer, 1960; Hanus, Sands & Bennett, 1967) required to inhibit bacterial growth are greater on agar plates than in liquid media.

While it is widely recognized that agar can provide a carbon source for microbial growth, it is generally believed that the carbon source is hexoses utilized through glycolysis. However, we present evidence suggesting that the fungus *Aspergillus nidulans* utilizes agar as a carbon source through C_2 metabolism rather than glycolysis. We have also observed antagonistic effects of agars on the utilization of lactic and succinic acids and of certain amino acids as carbon sources for growth by mutants with lesions in intermediary metabolism. These compounds support the growth of some acetate non-utilizing (*acu*) mutants of *A. nidulans* in liquid media, whereas there is no growth on the same media solidified with agar. Our data illustrate how the presence of agar in test media can generate both false positive and false negative results.

METHODS

Strains. The strains of *Aspergillus nidulans* used derived from the Glasgow stock and all were in the R21 genetic background, *pabaAr yA2* (requirement for *p*-aminobenzoic acid, yellow conidia). The origins of the various mutations and their associated enzyme lesions are given in Table 1.

Media. The defined minimal medium contained (g/l distilled water): KNO₃, 6.0; KCl, 0.52; KH₂PO₄, 1.52; and a trace elements solution (Armitt, McCullough & Roberts, 1976). The medium was brought to pH 6.5 and supplemented with *p*-aminobenzoic acid (1.0 mg l⁻¹). MgSO₄·7H₂O was added separately to 0.52 g l⁻¹.

All carbon sources, except aspartic acid, were prepared as stock solutions in distilled water, brought to pH 6.5 where necessary, and sterilized by autoclaving at 121 °C for 10 min, or, in the case of sucrose and galactose, 110 °C for 20 min. A dilute solution of aspartic acid was adjusted to pH 6.5 and incorporated into the final medium which was then autoclaved.

Media for plates were solidified with 1.5 % (w/v) Davis New Zealand agar and sterilized by autoclaving at 121 °C for 15 min. The carbon source was added aseptically from stock solution to the cooled molten medium immediately before pouring the plates; these were dried before stab inoculation with conidia.

Media for liquid culture, containing the wetting agent Tween 80 diluted 10⁻⁵ (v/v), were autoclaved at 121 °C for 15 min before adding MgSO₄ and carbon source from stock solutions. The medium was then distributed aseptically in 5.0 ml samples to capped 17 mm diam. test tubes. The cultures were inoculated with a standard loop of conidia and held at an angle during incubation on a rotary platform shaker.

Both plates and liquid cultures were incubated at 37 °C.

Sources of agar. The various agars were obtained from commercial suppliers. Electrophoretic quality agarose was from BDH Chemicals. Agars were washed by suspension followed by filtration using 10 vols chloroform, 10 vols methanol, three washes in 20 vols ethanol and six in 50 vols distilled water.

RESULTS AND DISCUSSION

Agar as carbon source for Aspergillus nidulans

A number of workers have found that agar provides a significant carbon source for the growth of *Aspergillus nidulans* (Hankinson, 1974; Fiddy & Trinci, 1975).

When conidia of *A. nidulans* were incubated in a defined liquid medium lacking added carbon source there was essentially no growth, although a small proportion of the conidia eventually germinated to produce a single hypha. However, when dilute suspensions of conidia were plated on the same medium solidified with agar but still with no added carbon source, a large proportion of the conidia formed isolated colonies which increased in diameter at a rate similar to that on glucose plates. However the resultant colonies were typically 'sparse', poorly conidiated with a low density of mycelium. Obviously, the organism was able to derive significant quantities of carbon from the agar. There was no apparent liquefaction of the agar around the colonies.

Hankinson (1974) found that mutants defective in the utilization of glycolytic carbon sources can grow on agar plates without added carbon source, although their growth may be inhibited when the non-utilized carbon source is added. This leaky growth of hexose non-utilizing mutants on agar alone is interpreted on the basis either of a mixture of different hexoses being present in agar or of alternative metabolic routes such as the phospho-pentose

Table 1. *The utilization of carbon sources by strains of Aspergillus nidulans for growth in liquid media or on agar plates*

The Table shows the relative growth responses to carbon sources supplied in defined liquid media or in the same media solidified with agar. Growth yields were scored visually for the amounts of mycelium formed in comparison with the wild-type strains R21. The carbon sources fell roughly into two groups with respect to growth of the wild type and these were scored + + + (sucrose, alanine, aspartate and glutamate) or + + (acetate, lactate, succinate). For each carbon source, yields were scored over a range: + + +; + +; +, poor yield; (+), mycelium visible; —, no growth. Cultures were scored after incubation for 1 day (sucrose), 2 days (acetate, lactate, alanine, aspartate, glutamate) or 3 days (succinate). Growth on agar plates with no added carbon source was apparent after 1 day's incubation: the Table shows growth at 3 days. While the growth of the wild type on each carbon source tested on agar plates or in liquid culture was similar, this does not imply that quantitative differences in yield would not be found.

The organic acids were tested with NO_3^- as nitrogen source; the same results were obtained when NH_4^+ was the nitrogen source. The same results were also obtained for each of the amino acids irrespective of whether the amino acid was supplied as the sole carbon and nitrogen source, or as the carbon source with added NO_3^- or NH_4^+ present.

Strains	Enzyme lesions*	Carbon sources for growth														
		None added	Sucrose (0.02 M)		Acetate (0.10 M)		DL-Lactate (0.1 M)		L-Alanine (0.05 M)		Succinate (0.1 M)		L-Aspartate (0.05 M)		L-Glutamate (0.05 M)	
			AP	LM OR AP	LM OR AP	LM	AP	LM	AP	LM	AP	LM	AP	LM	AP	
R21		+	+++	++	++	++	++	+++	+++	++	++	+++	+++	+++	+++	
<i>pycA3</i>	Pyruvate carboxylase	+	—	++	(+)	(+)		—	+	++	++	+++	++	++	++	
<i>pdhA1</i>	Pyruvate dehydrogenase	+	—	++	(+)	++		(+)	++	(+)	++	++	++	++	+++	
<i>acuA204</i>	Acetyl-CoA synthase	—	+++	—	++	—		+++	—	++	+	++	(+)	+++	+	
<i>facB2-101</i>	Not known	(+)	+++	(+)	++	—		+++	—	++	+	+++	(+)	+++	+	
<i>acuC203</i>	Not known	—	+++	—	++	—		+++	—	++	+	++	(+)	+++	+	
<i>acuD202</i>	Isocitrate lyase	—	+++	—	(+)	—		+++	—	++	—	+	—	+++	(+)	
<i>acuE201</i>	Malate synthase	—	+++	—	++	+		+++	++	++	+	+	—	+	+	
<i>acuK248</i>	Malic enzyme	—	+++	—	—	—		—	—	+	—	++	—	—	—	
<i>acuM2-301</i>	Malic enzyme	—	+++	—	+	—		+	—	+	—	++	—	+	—	
<i>acuF238</i>	PEP carboxykinase	—	+++	—	—	—		—	—	—	—	—	—	—	—	
<i>acuG223</i>	Fructose 1,6-diphosphatase	(+)	+++	(+)	+	(+)		+	+	+	—	(+)	(+)	+	—	
<i>acuL217</i>	Not known	—	+++	—	+	—		+	—	++	—	++	—	(+)	—	
<i>acuH206</i>	Not known	—	+++	—	++	—		++	—	+	—	++	—	++	+	
<i>acuJ211</i>	Not known	—	+++	—	—	—		—	—	—	—	(+)	—	++	+	
<i>acuN2-356</i>	Not known	—	+++	—	+	—		—	—	—	—	+++	—	+	—	

LM, liquid medium; AP, agar plate.

* For details of enzyme lesions, see: *pycA*, Skinner & Armitt (1972); *pdhA*, Romano & Kornberg (1969); *acu* strains, Armitt *et al.* (1976).

pathway. The implication that the carbon sources present in agar are either not hexoses or are not metabolized through glycolysis, appears to have been overlooked.

We found that glycolytic mutants lacking pyruvate dehydrogenase (*pdhA*) or pyruvate carboxylase (*pycA*) show similar phenomena (Table 1). These mutants did not grow on sucrose, glucose or galactose but grew well on other carbon sources such as acetate or glutamate. They grew on agar plates without added carbon source – though not so well as the wild-type did – but this growth was inhibited by a low concentration (1 mM or less) of sucrose or glucose. Acetate non-utilizing (*acu*) mutants, which grew well on sucrose or glucose, did not grow at all on agar plates with no added carbon source. Taken together these observations strongly suggest that *A. nidulans* utilizes agar as a carbon source not through glycolysis but through the pathways of acetate metabolism (Kornberg, 1966).

The data presented in Table 1 refer to Davis New Zealand agar but other commercial agars (Difco, Oxoid, Fisons) gave essentially the same results. We first thought that the carbon source was some contaminating material, but thorough washing of the agar with chloroform, ethanol and distilled water did not greatly diminish its capacity to support growth. It is therefore unlikely that the carbon source has low molecular weight or is a fatty acid (Sykes, 1956). We also considered the possibility that the organism derived C₂ compounds from residues substituted on the galactose units of the polymers, since many agarophytes contain D-galactose units bearing a pyruvic acid acetal, 4,6-*O*-(1-carboxyethylidene)-D-galactose, and these may constitute up to 3.0% of the polymer (Young *et al.*, 1971). However when electrophoretic quality agarose was used in plates with no other added carbon source, growth of the wild type, and *pycA* and *pdhA* strains was as good as on the commercial agars. Washing the agarose did not diminish the growth of the wild type or of the glycolytic mutants. The *acu* mutants did not grow on agarose plates.

It appears that *A. nidulans* utilizes agar by degradation of the polymers, although the amount of degradation is too small to cause liquefaction of the agar or to be detected as a change in the iodine staining reaction (Ng Ying Kin & Yaphe, 1972; Hofsten & Malmqvist, 1975). It is not clear how the agar polymers could be degraded to be utilized through the pathways of acetate metabolism (Kornberg, 1966). The occurrence of *exo*-glycoside α - and β -galactosidases in *Aspergillus niger* (Bahl & Agarwal, 1972) and of an extracellular *exo*- α -1,3-glucanase together with other glycosidases in *A. nidulans* (Zonneveld, 1972*a, b*) suggests that agar might be degraded by extracellular enzymes releasing terminal galactose residues. However, if galactose residues are the principal product of agar degradation, mutant strains unable to grow with galactose as carbon source should also be unable to grow on agar alone. This is clearly not the case with the *pycA* and *pdhA* mutants which are unable to utilize galactose, yet do grow on agar. Therefore it seems unlikely that galactose is the major product of agar degradation.

Ways in which agar can obscure growth tests for the utilization of potential carbon sources are illustrated in Table 1. Growth of the *pdhA* mutant, which requires a source of acetyl-CoA, on the lactate and the alanine test plates contradicts the expectation that this mutant cannot derive C₂ compounds from these carbon sources. The virtual absence of growth of this mutant in liquid media containing the same carbon sources strongly suggests that agar is supplying a source of acetyl-CoA. The inability of agar to enhance the growth of the *pycA* mutant, which requires C₄ dicarboxylic acids, on solid media containing alanine and lactate may indicate that the concentration of the compound derived from agar is not sufficient to induce the enzymes of the glyoxylate cycle (Kornberg, 1966). These findings also rationalize a puzzling feature concerning many of the revertants of *pycA* strains selected by their growth on sucrose agar plates (unpublished results). The majority of the revertants fail to

grow on sucrose in liquid medium and contain suppressor mutations of the *pycA* lesion. It now appears likely that the suppressor mutations may relieve carbon catabolite repression of the utilization of agar as a C source, possibly by derepressing the formation of extra-cellular enzymes for agar degradation (Zonneveld, 1972*b*).

In a medium containing a carbon source such as glucose but no added nitrogen source, agar supplies a significant source of nitrogen for the growth of *A. nidulans*. Thus similar difficulties to those discussed could occur when potential nitrogen sources are being tested on agar plates (Arst & Cove, 1973; Polkinghorne & Hynes, 1975). Under similar conditions there is barely any growth on agarose plates. The high sulphate content of agars could also complicate studies with mutants affecting sulphur metabolism.

*Agar inhibition of the utilization of certain carbon sources by
acu mutants in A. nidulans*

Our attention was drawn to this phenomenon by the failure of an *acuA* mutant to grow on lactate or alanine test plates while growing well in the same medium without agar. This result was not expected from the known lesion in acetyl-CoA synthetase in the mutant. Discrepancies were also found between growth tests done in liquid media and on agar plates for other strains (Table 1). Mutants defective in gluconeogenic functions, particularly the non-leaky *acuF* mutant lacking PEP carboxykinase (Armitt *et al.*, 1976), showed no growth on any of the carbon sources tested except sucrose. However, in several other cases, failure of *acu* mutants to grow on a particular carbon source in plates was not expected from knowledge of the enzyme lesions, for example the isocitrate lyase deficient *acuD* mutant on glutamate or succinate plates, and in each case the expected positive growth response was found in liquid media.

The reason for the lack of growth on agar media is not clear. It is conceivable that the very different physical environments may be important. Alternatively, it may be the presence of charged agaroproteins in the agar which causes these effects, although this seems unlikely since we found the same inhibitory effects in test plates made up with agarose, which is neutral. The hypothesis that agar, or its cleavage products, represses the utilization or uptake of other carbon sources could explain much of the data.

We do not know if the agar effect is specific to the *acu* mutants. We do, however, urge some caution in the interpretation of growth tests for the utilization of organic acids, and especially amino acids, done only on agar plates, and in the conclusions drawn about the effects of mutant lesions on their metabolism.

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