Agar as a Carbon Source in Relation to the Isolation of Lactose Non-utilizing Mutants of *Aspergillus nidulans*

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INTRODUCTION

We have described how agar provides a significant source of carbon for the growth of *Aspergillus nidulans* (Payton *et al.*, 1976) and shown that the inability of acetate non-utilizing (*acu*) mutants to grow on agar as sole carbon source suggests that agar is utilized by the organism through the pathway of acetate metabolism (Armitt *et al.*, 1976). We have also demonstrated that the presence of agar in test media can generate spurious growth responses; in particular, certain *acu* mutants do not grow on carbon sources such as lactate, alanine, succinate, aspartate, glutamate (Payton *et al.*, 1976) or lactamide (Payton, unpublished results) when agar is present in the medium. In contrast, the *acu* mutants do grow on these carbon sources when tested in liquid culture, unless the metabolic lesion in the mutant strain itself prevents growth on a particular carbon source.

These results prompt us to suggest a model for the growth of *A. nidulans* on agar in which the degradation of the agar polymers is effected by extracellular enzyme(s). Such a model is consistent with reports of extracellular enzymes from *A. nidulans* capable of degrading complex polysaccharides (Zonneveld, 1972*a, b*). We assume that the ability of agar to inhibit the utilization of other potential carbon sources requires at least the initial degradation of the agar polymers, though the precise mechanism by which the *acu* mutants are prevented from utilizing the alternative carbon sources is not understood. However, since the formation of extracellular enzymes by *Aspergillus* is usually catabolite-repressed (Bailey & Arst, 1975; Cohen, 1977), it is likely that the agar-degrading enzymes are also subject to such carbon catabolite repression. Thus one might expect that the utilization of carbon sources which are strong catabolite repressors, such as glucose or sucrose, will not be susceptible to 'agar-inhibition' due to repression of the synthesis of the enzyme(s) responsible for agar degradation. Conversely, the utilization of carbon sources which are weak catabolite repressors will exhibit 'agar-inhibition', that is, they will not support the growth of *acu* mutants when present in media solidified with agar.

If the hypothesis is correct, then mutant strains selected for no growth on carbon sources which are weak catabolite repressors may in some cases be analogous to the *acu* mutants. In particular, we thought it possible that certain strains isolated in this laboratory (Fantes & Roberts, 1973) and elsewhere (Gajewski *et al.*, 1972) as lactose non-utilizing mutants may not be defective in the catabolism of lactose itself but contain lesions in acetate metabolism.

METHODS

Strains. The strains of *Aspergillus nidulans* used were derived from the Glasgow stock and were in the R21 genetic background, *pabaA1 yA2* (requirement for *p*-aminobenzoic acid, yellow conidia). The mutants used and their associated enzyme lesions are given in Table 1.

Media. The defined minimal medium contained (g l⁻¹ in distilled water): KNO₃, 6.0; KCl, 0.52; KH₂PO₄, 1.52; and a trace elements solution (Armitt *et al.*, 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 were prepared as stock solutions in distilled water and sterilized by autoclaving at 110 °C for 20 min.

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.

Assay for isocitrate lyase activity. Mycelium was harvested by filtration under suction on Whatman no. 1 filter paper and washed with distilled water and 0.02 M-phosphate buffer at pH 7.0. The mycelium was broken by sonication (MSE 150 W ultrasonic disintegrator) for 30 s in ice-cold buffer and cell debris was removed by centrifuging at 12000 g for 20 min at 0 to 4 °C.

Isocitrate lyase (EC 4.1.3.1) was assayed spectrophotometrically at 37 °C by following the formation of glyoxylate phenylhydrazine at 324 nm. The assay mixture contained (in 1.0 ml final vol.) 25 µmol imidazole buffer pH 6.8, 5 µmol MgCl₂, 1 µmol EDTA, 4 µmol phenylhydrazine hydrochloride and 2 µmol DL-isocitrate.

RESULTS

Growth of acetate non-utilizing (acu) mutants on lactose

The carbon sources whose utilization was found to be sensitive to 'agar-inhibition' (Payton *et al.*, 1976) are all poor catabolite repressors (Bailey & Arst, 1975), which is consistent with the hypothesis developed above. However, these carbon sources are also metabolized gluconeogenically and this raised the question whether the utilization of a carbon source which is a poor catabolite repressor but which is metabolized by glycolysis rather than by gluconeogenesis would exhibit 'agar-inhibition'. To test this, we examined the growth of the *acu* mutants on glucose, galactose or lactose, in the presence or absence of agar (Table 1). While these sugars are all utilized by glycolysis (McCullough *et al.*, 1977), they differ in their effectiveness as carbon catabolite repressors. Glucose is a strong and galactose a moderate catabolite repressor, but lactose is a weak catabolite repressor in *A. nidulans* (Fantes & Roberts, 1973; Arst & Cove, 1973; Bailey & Arst, 1975). The data (Table 1) show that while growth of the *acu* mutants on glucose and galactose is the same whether tested in liquid culture or on agar plates, their growth on lactose is inhibited when agar is present. That is, the utilization of glucose and galactose escapes 'agar-inhibition' while the utilization of lactose does not; this is good evidence supporting our hypothesis.

Lactose non-utilizing mutants

A number of strains of *A. nidulans* unable to grow on lactose are known. For example, Gajewski *et al.* (1972) isolated 24 lactose non-utilizing mutants, only six of which totally failed to grow on lactose. These six mutants, which grew normally on glucose or galactose, all failed to grow on acetate whilst the remaining 18, which were leaky for growth on lactose, grew normally on acetate. In addition, five of the six strains did not utilize a number of other similar carbon sources, notably succinate. The model for 'agar-inhibition' suggests that these six strains were *acu* mutants.

In another study of lactose utilization by *A. nidulans*, Fantes (1972) sought mutants lacking β-galactosidase and isolated a number of strains defective in growth on lactose. Only one strain, designated *lac105*, showed all the characteristics expected of a mutant lacking β-galactosidase, i.e. no growth on lactose, normal growth on other sugars and very low β-galactosidase activity. However, strain *lac105* proved to be a double mutant containing one mutation causing low β-galactosidase activity (*bga* mutation) and another mutation with no effect on β-galactosidase activity, yet causing poor growth on lactose (*lac* mutation). Recombinant strains carrying only the *bga* mutation grew as well as the wild type on lactose solid media though they did not grow in liquid culture. That is, deficiency of β-galactosidase

Table 1. Growth of *A. nidulans* *acu* mutants on glucose, galactose or lactose

The results show the relative growth yields in response to carbon sources (0.02 M) supplied in defined liquid medium or in the same medium solidified with agar. Growth yields were scored visually for the amount of mycelium produced in comparison to the wild-type strain R21. For each carbon source, yields were scored over the range +++ (good yield), + and (+) (poor yields). Cultures were scored after 2 d. While the growth of the wild type on each carbon source on agar plates or in liquid culture was similar, this does not imply that quantitative differences would not be found. Enzyme lesions are described in Armitt *et al.* (1976).

Strain	Enzyme lesion	Carbon source for growth			
		D-Glucose or D-Galactose		Lactose	
		Agar plate	Liquid culture	Agar plate	Liquid culture
R21 (wild-type)	—	+++	+++	+++	+++
<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-bisphosphatase	+++	+++	+	+++
<i>acuL217</i>	Not known	+++	+++	(+)	+++
<i>acuH206</i>	Not known	+++	+++	(+)	+++
<i>acuJ211</i>	Not known	+++	+++	(+)	+++
<i>acuN2-356</i>	Not known	+++	+++	+	+++

apparently did not prevent the utilization of lactose. In the light of this result, two separate pathways were proposed for lactose utilization in *A. nidulans*; one requiring β -galactosidase activity and relatively unimportant during growth on lactose plates but necessary for growth on lactose in liquid culture, and an alternative pathway independent of β -galactosidase activity (Fantes & Roberts, 1973). With our present knowledge of the utilization of agar as carbon source, the growth of a strain lacking β -galactosidase on lactose plates is not surprising since it is due to utilization of the agar, rather than utilization of the lactose. This can occur since lactose does not repress the synthesis of the agar-degrading extracellular enzymes.

The nature of the *lac* mutation in the *lac105* strain was not studied further, though it was tentatively assigned to chromosome V by mitotic haploidization (Fantes, 1972). We felt that the *lac* mutation may be analogous to an *acu* mutation and have re-examined *lac105*.

Is the lac mutation an acu mutation?

Although the strain *pabaA1 yA2; lac* grew very poorly on lactose plates, it grew well on lactose in liquid culture, which is precisely the result obtained for the *acu* mutants. In addition, *lac* did not grow on certain gluconeogenic carbon sources in solid media, including acetate or alanine. Moreover, in liquid culture, *lac* did not grow on acetate but grew normally on alanine. This pattern of growth responses is identical to those we have previously described for several *acu* mutants (Payton *et al.*, 1976) and indicates that *lac* is an *acu* mutation. The *lac* mutation is located on chromosome V (Fantes, 1972) and could be in one of five possible *acu* gene loci, *acuH*, *A*, *D*, *G* or *N*. Two of these loci, *acuN* and *acuG*, can be ruled out since *acuN* mutants do not grow on alanine in liquid culture (Payton *et al.*, 1976) whereas *lac* does, and *acuG* mutants do not grow on glycerol (Armitt *et al.*, 1976) while *lac* does.

Two results prove that *lac* is a mutation at the isocitrate lyase structural gene locus (*acuD*) in chromosome V. First, cell-free extracts of *lac* grown on a mixture of sucrose and acetate

were found to contain no isocitrate lyase activity. Second, there was no growth on acetate of a heterokaryon established between *lac* and *acuD320*, indicating non-complementation. Complementation was found between *lac* and strains characterizing each of the other 12 known *acu* loci (Armitt *et al.*, 1976).

Thus the original strain *lac105* isolated by Fantes (1972) was a double mutant containing one mutation *bgaA* in a structural gene (linkage group III) for β -galactosidase (Fantes & Roberts, 1973), and a second mutation, originally called *lac*, in the structural gene locus *acuD* (linkage group V) for isocitrate lyase. The isolation of such a double mutant strain is not surprising since the mutagen was nitrosoguanidine and severe selection was applied in the screening programme.

DISCUSSION

The implications of the work described here are twofold. First, it is clear that 'agar-inhibition' of *acu* mutants is found not only on those carbon sources we reported previously (Payton *et al.*, 1976) but also on lactose, a glycolytic carbon source. This may well reflect that 'agar-inhibition' is common to all carbon sources which are weak carbon catabolite repressors. We are unable to explain how agar inhibits growth of *acu* mutants on alternative carbon sources, except to propose that the *acu* mutants accumulate a toxic metabolite from agar degradation (Payton *et al.*, 1976). However, it is certain that the selection of mutant strains unable to grow on agar plates containing carbon sources which are weak catabolite repressors may generate unexpected classes of mutant, specifically *acu* mutants.

Second, the demonstration that *lac* is actually an *acuD* mutant allele deficient in isocitrate lyase means that the contention that *A. nidulans* metabolizes lactose by two pathways (Fantes & Roberts, 1973) is no longer tenable.

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