Isolation of mutants deficient in acetyl-CoA synthetase and a possible regulator of acetate induction in *Aspergillus niger*

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Acetate-non-utilizing mutants in *Aspergillus niger* were selected by resistance to 1.2% propionate in the presence of 0.1% glucose. Mutants showing normal morphology fell into two complementation groups. One class of mutant lacked acetyl-CoA synthetase but had high levels of isocitrate lyase, while the second class showed reduced levels of both acetyl-CoA synthetase and isocitrate lyase compared to the wild-type strain. By analogy with mutants selected by resistance to 1.2% propionate in *Aspergillus nidulans*, the properties of the mutants in *A. niger* suggest that the mutations are either in the structural gene for acetyl-CoA synthetase (*acuA*) or in a possible regulatory gene of acetate induction (*acuB*). A third class of mutant in a different complementation group was obtained which had abnormal morphology (yellow mycelium and few conidia); the specific lesion in these mutants has not been determined.

Keywords: acetyl-CoA synthetase, propionate resistance, *Aspergillus niger*

INTRODUCTION

It has previously been reported in *Aspergillus nidulans* that spontaneous resistant sectors arose on 1.2% propionate medium, some of which were acetate-non-utilizing mutants, and it was suggested that propionate could be used as a general selection technique for isolating acetate-non-utilizing mutants in filamentous fungi (Sealy-Lewis, 1994). Three classes of acetate-non-utilizing mutant were obtained, in (i) *facA* (*acuA*), which encodes the structural gene for acetyl-CoA synthetase (ACS) (Apirion, 1965; Armitt *et al.*, 1976; Sandeman & Hynes, 1989), (ii) *facB* (*acuB*), a regulatory gene involved in the induction of ACS, enzymes of the glyoxylate bypass, acetamidase and NADP-isocitrate dehydrogenase (Apirion, 1965; Armitt *et al.*, 1976; Kelly & Hynes, 1977, 1982; Katz & Hynes, 1989; Todd *et al.*, 1997) and (iii) *acuO*, a gene which had not previously been described and whose function is unknown. At least two of the classes of mutant selected by resistance to propionate were also selected as being resistant to fluoroacetate (*facA* and *facB* mutants; Apirion, 1965). *acuO* has not been tested on fluoroacetate as a substrate. Although no mutants in *facC* were isolated as being

resistant to propionate, a previously isolated *facC102* mutant is also resistant to propionate (unpublished). In addition to the mutants described above, a mutant was also isolated that was resistant to propionate but was not defective in acetate metabolism; this was found to be either allelic with, or closely linked to *facA*. A general screen for propionate-resistant mutants in *A. nidulans* resulted in *Acu*[−] and *Acu*⁺ isolates with approximately equal frequency (unpublished); the latter class of mutant could be analogous to the fluoroacetate-resistant *Acu*⁺ mutants (*fan* mutants) isolated by Armitt *et al.* (1976).

The basis of the selection on propionate of the acetate-non-utilizing mutants was thought to be that propionate itself is not toxic, but is converted to a toxic product. Propionate is metabolized by the 2-methylcitrate pathway in *Saccharomyces cerevisiae* (Pronk *et al.*, 1994); this pathway was also found in 21 yeast species and six species of filamentous fungi when surveyed by Miyakoshi *et al.* (1987). The initial step of this pathway is the formation of 2-methylcitrate from propionyl-CoA and oxaloacetate. In a series of reactions similar to the normal tricarboxylic acid cycle reactions, 2-methylcitrate is converted to 2-methylisocitrate, which is then cleaved into pyruvate and succinate. Much less commonly, propionate was found to be metabolized through a modified β -oxidation pathway. Plants, humans and

Abbreviations: ACS, acetyl-CoA synthetase; ICL, isocitrate lyase.

insects utilize the methylmalonyl-CoA pathway (Wegener *et al.*, 1968; Rosenberg & Fenton, 1989; Halarknar *et al.*, 1985). The enzymes of the 2-methylcitrate pathway have been found in *Aspergillus niger*, but have not been confirmed in *A. nidulans* (Miyakoshi *et al.*, 1987). Whichever pathway is utilized, the initial step involves the conversion of propionate to propionyl-CoA. Thus, *facA* and *facB* mutants are resistant to propionate because they lack ACS. *facC* has recently been cloned and sequenced (Stemple, 1997) and encodes a cytoplasmic acetylcarnitine transferase. The role of *acuO* is still unknown.

A. nidulans is generally regarded as a model organism for studies on the molecular genetics and biochemistry of filamentous fungi. We thought that it would be of interest to use the propionate selection technique to identify mutations in comparable steps in acetate utilization in the commercially important *A. niger* and establish whether the regulation of the early steps of acetate activation is similar in the two organisms.

Selection for resistance to sodium propionate as the sole carbon source in *A. niger* did not yield any resistant mutants. However, in contrast to *A. nidulans*, *A. niger* was unable to grow on 1.2% propionate in the presence of 0.1% glucose, and acetate-non-utilizing mutants were obtained on this medium. We present evidence for the isolation of mutants in three complementation groups, including probable mutations in the structural gene for ACS and a possible regulatory gene.

METHODS

Strains and genetic analysis. The wild-type strain was *cspA1*; *nicA1* having short conidiospores and a requirement for nicotinic acid. The strain used for haploidization analysis was N827: *fwA1*; *hisD4*; *bioA1* *lysA7*; *leuA1*; *pabA1*; *cnxCS*; *trpB2* (kindly provided by Dr Klaas Swart, Department of Genetics, Wageningen Agricultural University, NL). The markers are described by Bos *et al.* (1988, 1993). The medium on which *A. niger* was grown was the same as that described for *A. nidulans* by Pontecorvo *et al.* (1953) and McCully & Forbes (1965), and modified by Cove (1966). The method used for assigning markers to linkage groups has been described by Bos *et al.* (1988, 1993).

Selection of mutants resistant to propionate. After UV mutagenesis, conidia were plated onto medium containing 1.2% (w/v) sodium propionate with or without 0.1% (w/v) glucose.

Growth of mycelia for enzyme assays. Mycelia were grown in shaking cultures for 18 h at 37 °C on 20 mM sucrose, 5 mM urea medium with appropriate supplementation. After filtration, mycelia were transferred for a further 6 h to medium containing 100 mM sodium acetate (induced) or 50 mM glucose (glucose repressed) as described by Armitt *et al.* (1976). Extracts were made by grinding the mycelium with sand before the addition of extraction buffer (Armitt *et al.*, 1976) and the extracts were centrifuged at an average of 20000 g for 30 min.

Enzyme assays. The acetyl-CoA synthetase assay was modified after the method of Berg (1956). Crude extracts were

prepared in 40 mM potassium phosphate buffer, pH 7.4. Each 600 µl reaction contained: 10 mM ATP, 0.1 mM coenzyme A, 200 mM hydroxylamine hydrochloride, 10 mM magnesium chloride and 10 mM potassium acetate in 40 mM potassium phosphate buffer, pH 7.4. The reaction was started by the addition of 15–120 µl crude extract and allowed to proceed for 20–40 min before the reaction was stopped by the addition of 900 µl acidified ferric chloride solution. Precipitated protein was removed by centrifugation and the absorbance was measured at 540 nm.

Isocitrate lyase was assayed according to the method of Armitt *et al.* (1976). The protein concentration was estimated by the microbiuret method (Goa, 1953).

RESULTS

Isolation of mutants resistant to propionate and glucose

UV irradiated conidia (70% kill) from the *cspA1*; *nicA1* strain were plated onto minimal medium with 1.2% sodium propionate as the sole carbon source as well as onto medium containing 0.1% glucose + 1.2% propionate. No resistant colonies were obtained on the propionate medium (total 1.9×10^8 viable conidia screened), but on the propionate + glucose medium, resistant colonies arose with a frequency of 1 in 4.7×10^5 viable conidia. One hundred and forty mutants were originally isolated; these were classified into three groups on the basis of their morphology on complete medium. Class I mutants had a morphology that was indistinguishable from the wild-type strain; the majority of resistant mutants were of this type. The remaining two classes (comprising a total of eight mutants) had abnormal morphology compared to the wild-type; these abnormalities resulted in sparse or no conidiation and a strong yellow colouration of the mycelium. Forty-eight mutants (42 of normal morphology and six of abnormal morphology) were purified so that isolates arose from a single conidium on fresh propionate + glucose medium; these purified isolates were further tested at 37 °C on medium containing 1.2% sodium acetate and 5 mM urea. All the mutants grew less well than the wild-type on acetate medium, although in the case of the mutants with morphological abnormalities and where conidiation was poor, it was difficult to assess the degree of impairment. *A. niger*, in common with *A. nidulans*, shows very weak growth on agar plates in the absence of any added carbon source. Armitt *et al.* (1976) concluded that for *A. nidulans*, the carbon source in the agar was lipid or compounds metabolized via C_2 units, since this growth was abolished in most of their *Acu*⁻ strains. On agar medium lacking a carbon source, the diameter of the colony increases at about the same rate as when grown on glucose medium, but the density of the colony is drastically reduced and there is no conidiation. The growth of the class I propionate-resistant mutants was no better on acetate medium than on medium lacking a carbon source.

Twenty-five of the morphologically normal class of mutant and six morphologically abnormal mutants were

Table 1. Classification of propionate-resistant mutants

Class/morphology	Mutant*	Gene designation	Linkage group
Class I; wild-type morphology	1.1, 7.1, 42.1, 43.1, 45.1, 47.1, 49.1, 50.1, 98.1, 105.1, 127.1, 131.1, 206.1, 213.1, 214.1, 220.1, 223.1, 235.1, 241.1, 251.1, 256.1, 258.1	<i>acuB</i> ; fails to complement 98.1	VIII
Class I; wild-type morphology	117.1, 264.1, 255.1	<i>acuA</i> ; complements 98.1	Unknown
Class II†; pale yellow mycelium, very few conidia	90.1, (129.1)	<i>acuC</i> ; 90.1 complements 98.1 and 117.1	V
Class III†; bright yellow mycelium, no conidia	(133.1), (134.1), 62.1, 91.1	<i>acuD</i> ? 62.1 and 91.1 complement 98.1 and 117.1; may be the same gene as 90.1	Unknown

* The mutants in parentheses are assigned to a group on the basis of their morphology, but complementation tests have not been performed.

† Class II mutants show pale yellow mycelia after 2 d incubation at 37 °C. Conidiation occurs slowly if left on the bench. Class III mutants show bright yellow mycelia and no conidia at 37 °C after 2 d incubation; a few scattered conidia are formed after prolonged incubation on the bench.

Table 2. Growth properties of the wild-type and propionate-resistant mutants

Growth is represented on a scale of 1–5. Growth scores are not necessarily comparable between plates. The growth media were: CM (complete medium); SC (1% glucose); –C (no carbon source); Ac (1.2% acetate); Prop (1.2% propionate); Prop + G (1.2% propionate + 0.1% glucose); Eth (1% ethanol); But (1.2% butyrate) and Tween (0.5%). In all cases the nitrogen source was 5 mM urea.

Strain	Growth on:								
	CM	SC	–C	Ac	Prop	Prop + G	Eth	But	Tween
<i>acuB98.1</i>	5	5	1–	1–	0	1*	1	1	4
<i>acuA117.1</i>	5	5	1–	1–	0	1*	1	1	4
<i>acuC90.1</i>	5	5	1	2	0	1*	1+	0	3
<i>acuD91.1</i>	3	3	1	2	0	1*	2	0	2
Wild-type	5	5	1	3	0	0	2+	1	4

* Growth on Prop + G was inhibited (colony diameters were reduced) but is indicated as 1.

selected for detailed analysis by complementation tests (see below) and further growth tests (Tables 1 and 2). The selected mutants were tested on the following carbon sources: 1.2% propionate, 1.2% propionate + 0.1% glucose, 1.2% sodium acetate, 1% ethanol, 0.5% Tween 80 (polyoxyethylenesorbitan monooleate), 0.1 M butyrate, and on medium lacking a carbon source (Table 2). In each case the nitrogen source was 5 mM urea. None of the mutants showed any growth on propionate medium in the absence of glucose (not even the weak growth seen on medium lacking a carbon source). Growth of the propionate-resistant mutants selected on

propionate + glucose was poor, but since there was no growth at all of the wild-type, the colonies were easy to identify in the original screen. This growth can be best interpreted as relief of the toxic effects of propionate in the medium, allowing utilization of the glucose. All the mutants grew less well than the wild-type on ethanol medium, although the mutants in class III (91.1, 62.1, 133.1 and 134.1) showed the strongest growth (Table 2). The class I mutants (which comprised two genes, see below) grew as well as the wild-type on Tween 80, but the class II and III mutants showed some growth reduction. Butyrate was a very poor carbon source for

the wild-type and growth was only observed after a week of incubation; the class II and III mutants did not grow on butyrate, while class I mutants grew as well as the wild-type. In general, the morphological mutants were hard to work with because it proved difficult to construct diploids for haploidization/complementation analysis.

Characterization of mutants

All the mutants described below were recessive to the wild-type *Acu*⁺ strain in a diploid with the multi-marked strain, N827. A diploid between one of the class I mutants, 98.1, and N827 was haploidized and *acu98.1* was found to map to linkage group VIII (data not shown). A recombinant isolate from the haploidization 98.1:N827 (*fwA1; bioA1 lysA7; leuA1; cnxC5; acu98.1*) was used for complementation tests with the other *Acu*⁻ mutants isolated. Complementation tests were performed on acetate medium and propionate+glucose medium. All the putative diploids isolated for these tests were checked to make sure that the volume of the conidia was characteristic of the diploid (see Bos *et al.*, 1988, 1993).

The majority of the mutants failed to complement mutant 98.1 (i.e. they failed to grow on acetate medium and were resistant on propionate+glucose medium) (Table 1). Another mutation of this complementation group, *acu105.1*, was assigned to linkage group VIII on the basis of haploidization analysis with N827. The mutants tested that complemented 98.1 were 117.1, 264.1 and 255.1, which were class I mutants with no morphological abnormalities. Mutants 90.1, 62.1 and 91.1 also complemented the 98.1 tester strain. It was not possible to make diploids with 129.1, 133.1 and 134.1 and therefore it has not been possible to characterize these mutants. Diploids were made between 117.1 and N827, 90.1 and N827, and 91.1 and N827. Subsequent haploidization analysis assigned *acu90.1* to linkage group V, although, presumably because of the extreme morphology associated with *acu90.1*, no *Acu*⁻ segregants were isolated after haploidization. Repeated analyses of segregants from the 117.1:N827 and 91.1:N827 diploids failed to assign these *Acu*⁻ mutants to a linkage group. However, for both 91.1 (*bioA1 lysA7; pabA1 cnxC5 acu91.1*) and 117.1 (*bioA1 lysA7; trpB2; acu117.1*), recombinant genotypes were isolated from the haploidizations that were utilized in further complementation analyses. Mutant 117.1 failed to complement 255.1 and 264.1, making it likely that these contain mutations in the same gene. We were aware that the complementation between *acu98.1* and *acu117.1* could have been a result of inter-allelic complementation and we therefore looked at complementation of *acu117.1* with other alleles in the class that failed to complement *acu98.1*, namely *acu50.1* and *acu105.1*; again complementation was seen, so we believe that the observed effect is unlikely to be due to inter-allelic complementation. Because *A. niger* lacks a sexual cycle, it is not possible to confirm by a cross to the wild-type that the *Acu*⁻ mutant phenotype is the result of a single

Table 3. Relative specific activities of ACS and ICL in wild-type and *Acu*⁻ mutant strains

All activities are expressed relative to the wild-type strain transferred from 0.02 M sucrose (Suc) to 100 mM acetate medium (Ac) or to 50 mM glucose (Glc). All determinations were performed at least twice, and standard error values are shown. NT; not tested. The wild-type activity induced on acetate (six determinations) was equivalent to a specific activity of 192.4 nmol product min⁻¹ (mg protein)⁻¹ (± 9.9) for ACS and 260.3 ± 8.5 nmol product min⁻¹ mg⁻¹ for ICL.

Strain	Transfer	Relative specific activity (%)	
		ACS	ICL
Wild-type	Suc $\rightarrow$ Ac	100	100
Wild-type	Suc $\rightarrow$ Glc	11.1 $\pm$ 0.26	2.7 $\pm$ 0.7
<i>acuB98.1</i>	Suc $\rightarrow$ Ac	45.3 $\pm$ 2.9	22.5 $\pm$ 1.0
<i>acuB50.1</i>	Suc $\rightarrow$ Ac	50.7 $\pm$ 10.6	27.0 $\pm$ 3.4
<i>acuA117.1</i>	Suc $\rightarrow$ Ac	4.7 $\pm$ 1.97	89.7 $\pm$ 6.2
<i>acuA255.1</i>	Suc $\rightarrow$ Ac	1.7 $\pm$ 0.58	70.5 $\pm$ 4.5
<i>acuA264.1</i>	Suc $\rightarrow$ Ac	2.2 $\pm$ 0.5	NT
<i>acuC90.1</i>	Suc $\rightarrow$ Ac	85.8 $\pm$ 3.75	59.0 $\pm$ 4.5
<i>acuD91.1</i>	Suc $\rightarrow$ Ac	28.2 $\pm$ 3.7	37.3 $\pm$ 5.0

gene mutation. However, in the haploidization analyses, the propionate-resistant phenotype always segregated with the *Acu*⁻ phenotype.

It is consistent with the biochemical data (see below) that the complementing groups exemplified by *acu98.1* and *acu117.1* are mutations in different genes. *acu117.1* complemented *acu90.1*, *acu91.1* and *acu62.1*. It has not been possible to construct the appropriate diploids in pairwise combinations between mutants 90.1, 91.1 and 62.1 to look at the complementation between these *Acu*⁻ mutants. All the mutants involved have morphological abnormalities which fall into two classes, although we cannot say whether they are all mutations in the same gene. They are, however, distinct from the complementing groups represented by *acu98.1* and *acu117.1*. Our data suggest that the *Acu*⁻ mutants isolated represent mutations in at least three genes.

Assay of enzyme activities in *Acu*⁻ mutants

Mycelia grown from mutants in the representative classes together with the wild-type control were grown on sucrose medium and transferred to 100 mM sodium acetate medium or 50 mM glucose medium. Extracts made from the mycelium were assayed for ACS and ICL (one of the enzymes of the glyoxylate bypass) activity (Table 3).

DISCUSSION

We have previously isolated mutants of *A. nidulans* in *facA* (defective in ACS) and *facB* (regulatory gene involved in acetate induction) by selection for resistance

to 1.2% propionate. In *A. niger*, analogous mutants were not selected by this technique, but were obtained after selection on 1.2% propionate + 0.1% glucose medium. In *A. nidulans*, the wild-type strain was able to grow on propionate + glucose medium, presumably because ACS is completely repressed in the presence of glucose (Kelly & Hynes, 1977; Sandeman & Hynes, 1989) and the wild-type is therefore insensitive to the toxic effects of propionate. In contrast, *A. niger* shows quite high levels of ACS in the presence of 0.1% glucose (Table 3) and therefore propionate would be converted to its toxic product, and the wild-type would be sensitive on propionate + glucose medium. The reason for the inability to select *A. niger* mutants resistant to 1.2% propionate is not clear.

The *A. niger* mutants that were selected on propionate + glucose medium were divided into four classes on the basis of their morphology and complementation tests; these represent mutations in at least three genes (Table 1). The mutants of the class which includes *acuA117.1* lack ACS activity, but have high levels of ICL (Table 3). These mutants are likely to be affected in the structural gene for ACS; we have called this class of mutant *acuA*. The fact that the *A. niger* ACS⁻ mutants have high levels of ICL suggests that acetate is the natural inducer, a situation that has been previously reported for *A. nidulans* (Hynes, 1977) and *Aspergillus terreus* (Das & Sen, 1983). The largest class of mutant, of which *acuB98.1* is a member, has reduced levels of ACS and ICL and is more similar to the regulatory mutant, *facB*, described in *A. nidulans*. We have called this class of mutant *acuB*. However, the *acuB* mutants in *A. niger* show quite high levels of ACS and ICL and we can only conclude that other enzymes in the glyoxylate pathway are more severely affected or that the levels of enzymes involved in growth on acetate have to be high to sustain growth on this poor carbon source. It has been shown that *A. niger* contains a regulatory gene of similar function to the *A. nidulans facB* gene, since the cloned *facB* gene has been isolated from *A. niger* by nucleic acid hybridization, using the *A. nidulans facB* gene as a probe. The *A. niger facB* homologue can substitute for the *A. nidulans facB* function in a null *facB* mutant in *A. nidulans* (Todd *et al.*, 1997). It had previously been reported that although *A. niger* does not contain a gene for acetamidase (*amdS*), the *amdS* gene from *A. nidulans* can function in *A. niger* and is inducible by acetate in a *facB*-dependent manner, suggesting that the *facB* homologue is required for acetate induction (Davis *et al.*, 1988).

The other mutants isolated, *acuC90.1* and *acuD91.1*, which may be mutations in the same gene, produce gross morphological alterations; because of this it is difficult to say whether they are really unable to grow on acetate. *acu91.1* shows impaired levels of ACS and ICL compared with the wild-type strain, while *acu90.1* shows quite high levels of ACS but lower levels of ICL (Table 3). Both mutants fail to grow on medium containing butyrate but they show some growth on medium containing Tween 80 (Table 2). The behaviour of these

strains could simply be a result of their aberrant morphology. Both *acuC90.1* and *acuD91.1* show yellow pigmentation of the mycelium and sparse conidiation, a property that has been described for mutations in *creA* from *A. niger* that map on linkage group IV (Ruijter *et al.*, 1997) and was thought to be due to derepression of the biosynthesis of a secondary metabolite. The *acuA* and *acuB* mutants, at least, grow on fatty acids and so their lesions are unlikely to be in the glyoxylate cycle, which would be required for fatty acid breakdown (Kawasaki *et al.*, 1995).

No propionate-resistant mutants that were able to grow on acetate but which had normal morphology were isolated in *A. niger* (analogous to the *fan* mutants in *A. nidulans*). In a general screen for mutants resistant to propionate in *A. nidulans* after UV mutagenesis, of 19 resistant mutants selected at random, 12 were able to grow on acetate medium and seven were *acu*⁻ mutants (unpublished). If the class of mutant that is propionate resistant but able to grow on acetate occurred with the same frequency in *A. niger*, we should have isolated such mutants. The inability to isolate *Acu*⁻ mutants on propionate alone and the failure to isolate propionate-resistant *Acu*⁺ mutants seems to be a fundamental difference between *A. nidulans* and *A. niger*. Propionate is a well-known antimicrobial compound with a long history of use as a preservative in foods and pharmaceuticals, although its exact mode of action is not fully understood (Russell & Chopra, 1990). The toxic intermediate that is responsible for inhibition in *A. niger* and *A. nidulans* cannot be determined from this study, but the concentration of propionate used to isolate mutants is high (125 mM) and as propionate is a substrate for ACS, high levels of propionyl-CoA are likely to accumulate. In *Neurospora crassa*, acetyl-CoA is known to be toxic during growth on high concentrations of acetate, since strains that lack acetyl-CoA hydrolase fail to grow, although growth is normal on ethanol or on low concentrations of acetate (Connerton *et al.*, 1992). If propionyl-CoA builds up in the mycelium, this might easily be toxic to the cell, either because it cannot be converted to propionate and CoA by the hydrolase or because the propionyl-CoA might inhibit any acetyl-CoA-dependent enzymes essential for growth.

Chattopadhyay *et al.* (1980) isolated acetate-non-utilizing mutants in *A. niger* after mutagenesis and replica plating onto acetate medium, and obtained two classes of mutant, neither of which corresponds to the mutants selected in this study. It seems likely that the selection method using propionate is widely applicable to filamentous fungi, although it may be necessary to include glucose in the selection medium for some fungal species. *A. niger* does not have a sexual cycle, but using the parasexual cycle it has been possible to obtain recombinant *acu* mutant genotypes and assign mutants to complementation groups. The isolation of acetate-requiring mutants in *A. niger* will aid comparative studies on the regulation of acetate utilization in *A. nidulans* and *A. niger*.

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