Derepression of the glyoxylate cycle in mutants of *Neurospora crassa* accelerated for growth on acetate

P. T. Chaure¹ and I. F. Connerton²

Author for correspondence: I. F. Connerton. Tel: +44 1734 357000. Fax: +44 1734 267917.
e-mail: IAN.CONNERTON@BBSRC.AC.UK

¹ Department of Microbiology, University of Reading, Whiteknights, PO Box 228, Reading RG6 2AJ, UK

² Institute of Food Research, Protein Engineering Department, Earley Gate, Whiteknights Road, Reading RG6 2EF, UK

Two spontaneous allelic mutations have been isolated with the unusual semi-dominant phenotype of faster-than-wild-type growth on acetate as sole carbon source. The mutants were designated *Aag-1* (accelerated acetate growth) and mapped on linkage group II. Upon re-isolation of both the *Aag-1* alleles from repeated back-crosses to wild-type, between 1 and 6% of the progeny were found to be *acu* (acetate non-utilizing) mutants. Ten of these were selected for heterokaryon complementation analysis with known *acu* mutants; nine proved to be new alleles of *acu-5* (deficient in acetyl-CoA synthetase), and one was a new acetate non-utilizing class, designated *acu-14*. Although the *Aag-1* mutants clearly have no acetate-growth-related enzyme deficiencies, they did exhibit significant constitutive enzyme levels for acetyl-CoA synthetase and the glyoxylate cycle enzymes (isocitrate lyase and malate synthase) on the non-inducing carbon source, sucrose. The derepression was restricted to these enzymes, as representative enzymes from other carbon-assimilatory pathways remained repressed and subject to carbon catabolite repression. Northern blot analysis of the mRNA levels of acetyl-CoA synthetase and the glyoxylate cycle enzymes from the mutants demonstrated the derepression to occur at the level of transcription. These data suggest that the physiological explanation for the accelerated acetate growth phenotype lies in the standing levels of the acetate-assimilatory enzymes, which enable the mutants to forgo some of the normal time required for adaption to growth on acetate.

Keywords: *Neurospora crassa*, ascomycete, glyoxylate cycle, acetate utilization

INTRODUCTION

Fungi utilize acetate as a sole carbon source via the action of acetyl-CoA synthetase. The resulting acetyl-CoA is incorporated into the glyoxylate cycle, which serves to replenish the tricarboxylic acid (TCA) cycle in the absence of glycolysis (Kornberg, 1966). This involves two reactions catalysed by the isocitrate lyase and malate synthase, which bypass the two decarboxylation steps of the TCA cycle. In addition to the enzymes of the glyoxylate cycle, phosphoenolpyruvate carboxykinase and NADP⁺-malic enzyme provide the metabolic links necessary for gluconeogenesis.

In a few species acetate non-utilization has been used as a criterion for the selection of biochemical mutants, including the filamentous ascomycetes *Aspergillus nidulans* (Armstrong *et al.*, 1976) and *Neurospora crassa* (Flavell & Fincham, 1968a, b), together with the basidiomycete *Coprinus cinereus* (Casselton & Casselton, 1974) and the yeast *Yarrowia lipolytica* (Barth, 1985). Flavell & Fincham (1968a, b) first isolated acetate non-utilizing mutants from *N. crassa*. These fell into seven distinct complementation groups mapping to seven independent loci. Three of these groups had single enzyme deficiencies: *acu-3* and *acu-5* lacked isocitrate lyase and acetyl-CoA synthetase, respectively, whereas *acu-6* was deficient in phosphoenolpyruvate carboxykinase, which links gluconeogenesis with the primary acetate catabolic enzymes (Beever, 1975). Later, Rao & Debusk (1977) reported an acetate non-utilizing permease-deficient mutant which was designated *acp⁻ⁱ*.

Abbreviations: RIP, repeat induced point mutation; TCA, tricarboxylic acid.

Using mRNAs expressed during the adaptation of *Neurospora* mycelia from sucrose to acetate as hybridization probes, Thomas *et al.* (1988) isolated genomic clones involved in acetate utilization. Two of these clones were identified by their ability to complement the *N. crassa* acetyl-CoA synthetase mutant (*acu-5*) and the *A. nidulans* malate synthase mutant (*acuE*). A third clone was used to disrupt the resident gene using the RIP (Repeat Induced Point mutation) phenomenon of *N. crassa* and to generate a novel *acu* mutant, *acu-8* (Marathe *et al.*, 1990). RIP is a pre-meiotic phenomenon in *N. crassa*, the effect of which is to cause extensive cytosine methylation and GC-AT transitions in duplicated DNA sequences (Selker *et al.*, 1987). Therefore, transformed DNA sequences that duplicate resident genomic regions will trigger the process during a sexual cross and result in the disruption of the participating sequences. Later, *acu-8* was found to represent a null allele deficient in acetyl-CoA hydrolase (Connerton *et al.*, 1992). Subsequently, the malate-synthase-deficient class was represented by RIP mutants and designated *acu-9* (Connerton, 1990).

N. crassa mutants falling into a further four acetate non-utilizing complementation groups (*acu-10* to *acu-13*) were isolated by Owen *et al.* (1992) on the basis of resistance to the antimetabolite fluoroacetate. In the fellow ascomycete *A. nidulans*, three fluoroacetate-resistant/acetate non-utilizing classes have been isolated (Apirion, 1965). Of these, *facA* is the structural gene for acetyl-CoA synthetase (Armstrong *et al.*, 1976; Connerton *et al.*, 1990), and *facB* has been shown to prevent normal induction of the glyoxylate cycle (Armstrong *et al.*, 1976; Katz & Hynes, 1989). However, unlike the situation in *A. nidulans* none of the *N. crassa* fluoroacetate-resistant classes proved to be deficient in acetyl-CoA synthetase, or to have phenotypes affected in the regulation of the glyoxylate cycle. Analogous mutants to the latter regulatory class have also been reported in two other fungal species: the *acu-1* mutations of *C. cinereus* (Macanochie *et al.*, 1992), and the *GPR1* mutations of *Y. lipolytica* (Kujau *et al.*, 1992). Two further mutants have been reported in *A. nidulans* that specifically cause the derepression of isocitrate lyase on non-inducing carbon sources (McCullough & Roberts, 1980; McCullough & Shanks, 1993). The *icl^cA* and *icl^cB* mutations act synergistically and are not affected by the presence of the *facB* mutation, implying that isocitrate lyase in *A. nidulans* is also subject to an alternative regulatory circuit independent of acetate induction.

It is perhaps surprising that none of the orthodox acetate non-utilizing *Neurospora* mutants have been shown to have regulatory effects on the expression of the glyoxylate cycle enzymes. In this paper we report the genetic and biochemical characterization of mutants of *N. crassa* that demonstrate a novel semi-dominant accelerated acetate growth phenotype and specific constitutive derepression of the glyoxylate cycle enzymes on non-inducing carbon sources such as sucrose.

METHODS

Strains. The *N. crassa* strains used in biochemical and heterokaryon tests were wild-type 74 *A/a* (ST *A/a*) and *acp⁻¹* (Rao &

Debusk, 1977); *acu-1* (JI48), *acu-3* (JI38), *acu-5* (JI18; 94-78), *acu-6* (JI31) and *acu-7* (JI36) (Flavell & Fincham, 1968a, b); *acu-8* (T27-1-8) (Marathe *et al.*, 1990); *acu-9* (T11-1) (Connerton, 1990); *acu-10* (S10-3), *acu-11* (S30-12), *acu-12* (S10-4) and *acu-13* (S30-9) (Owen *et al.*, 1992); *trp-3* (FGSC4093), *pyr-4* (FGSC4030) and *A/a am¹³²* (Kinnaird *et al.*, 1982). The linkage group tester strain *alcay csp* was used in initial mapping studies (Perkins, 1964): *A/a T(I; II)4637 al-1; T(IV; V)R2355, cot-1; T(III; VI)1, ylo-1; csp-2* (FGSC3661/3434). The secondary testers for linkage-group determination were LGI and LGII *a al-1, arg-5* (FGSC1206); LGIV and LGV *a cot-1, al-3* (FGSC1244); LGIII and LGVI *a trp-1, ylo-1* (FGSC1208); and LGVII *a nic-3, met-7, arg-10* (FGSC4142).

Media. Vogel's minimal medium (Vogel, 1956) was supplemented with 2% (w/v) sucrose or with one of the following alternative carbon sources: 40 mM sodium acetate, 40 mM ethanol, 40 mM quinate, 0.5% Avicel, 0.5% oat spelt xylan. Solid medium contained 1.5% (w/v) agar (Difco), 0.5% (w/v) fructose and glucose (BDH) as carbon source together with 2% (w/v) L-sorbose (Sigma) for induction of colonial growth (Tatum *et al.*, 1949). When using acetate as carbon source colonial growth was induced with 0.2% L-sorbose. Vogel's fluoroacetate medium contained 0.5 M sodium fluoroacetate (Aldrich/Sigma) supplemented with 0.5% fructose and glucose and 0.2% sorbose. Tryptophan (1 mM), uracil/uridine (3 mM) and glutamate (3 mM) were used as supplements for the *trp-3*, *pyr-4* and *am* mutants, respectively. Vogel's medium supplemented with 20 mM glycine was used to counter-select *am* mutants. Methionine (1 mM), arginine (1 mM) and niacinamide (2 µg ml⁻¹) were used as supplements for the linkage group VII markers.

Growth tests. A loopful of conidia was plated onto agar containing the appropriate growth medium including the paramorphogen sorbose to produce colonial growth. Growth was normally scored after 3 d incubation at 30 °C. However, when scoring the accelerated acetate growth phenotype the plates were checked at 1, 2 and 3 d to ensure the demarcation between mutant and wild-type strains. Growth was scored following overnight incubation at 30 °C when using media without sorbose, except when scoring for *cot*, when 37 °C was used. For scoring in liquid medium, 0.1 ml of a 10⁶ ml⁻¹ conidial suspension was used to inoculate 5 ml medium, and growth was scored after standing for 2–3 d. Growth curves were generated by inoculating multiple 50 ml aliquots of Vogel's sucrose or acetate medium with approximately 10⁸ conidia and harvesting duplicate samples at timed events. The mycelial yields of these samples were determined as dry weights and averaged.

Crossing and revival of ascospores. 1 × Westergaard and Mitchell's crossing medium (Westergaard & Mitchell, 1947) was used for sexual crosses. Petri dishes containing discs of 3MM Whatman filter paper (cellulose) as a carbon source were used to increase the fertility of crosses. Ascospores were collected from the Petri dish lid after release and after heat-shock at 60 °C they were germinated on Vogel's sucrose medium plus the appropriate supplements. A random sample of germinated ascospores was selected and transferred to slopes of a similar medium and incubated further until abundant conidia were present. Conidia from each isolate were then tested for their ability to grow on selective media with sorbose.

Complementation tests. Drops of dense conidial suspensions of the mutants to be tested were added to 5 ml liquid medium and incubated at 30 °C until many new conidia had formed on the mycelial growth. Of these new conidia some were presumed to be heterokaryotic and were then tested for their ability to

grow on appropriate media. Single colonies of these could be picked and their growth on further selective media was scored.

Enzyme assays. Enzyme activities were determined from crude extracts of wild-type and mutant strains. Mycelial pads were grown in $1 \times$ Vogel's sucrose medium for 16 h and induced for 8 h on inducing medium at 30 °C with shaking. Crude enzyme extracts were then prepared by grinding the mycelial pads with chilled 0.05 M sodium phosphate buffer (pH 7.2) and acid-washed sand. Supernatants were retrieved after centrifugation in a microcentrifuge for 1 min at 13000 r.p.m. and stored on ice for use in enzyme assays and protein determination. Protein concentrations were determined using Coomassie Brilliant Blue G-25 dye-binding. Reagent volumes were used such as to enable the assay to be run in a microtitre plate in 200 μ l aliquots using an ELISA plate reader calibrated at 720 nm. The subsequent enzyme assays were done as described by Dixon & Kornberg (1959) and detailed in Owen *et al.* (1992). Glucosidase and xylanase assays were performed on culture supernatants following 4 d growth on Avicel or oat-spelt xylan.

Northern transfers. RNA was extracted from *N. crassa* mycelium according to the method of Sokolovsky *et al.* (1990) using LiCl. RNA was electrophoresed in formaldehyde denaturing agarose gels according to the method in Maniatis *et al.* (1982) with $1 \times$ MOPS, blotted on to Hybond-N nylon filters (Amersham) and hybridized to labelled ('Multiprime labelling'; Amersham) DNA probes. The resulting autoradiographs obtained with different radiolabelled probes were scanned using a laser densitometer and scaled to each other. The intensities of the relative signals were then recorded.

RESULTS

Isolation of mutants

To check the culture medium and the wild-type parent (ST4) for a routine cross, a conidial suspension was plated out in serial dilutions onto Vogel's acetate containing 0.2% L-sorbose. This medium usually supports visible colonial growth of acetate-utilizing strains after 3 d at 30 °C. Unexpectedly, a vigorously growing colony appeared after 24 h, and other colonies appeared after 2–3 d growth. The colony was picked and subcultured on Vogel's sucrose medium and re-tested for growth on acetate plates, where it continued to grow faster than several acetate-utilizing laboratory strains. Approximately 10^5 conidia of wild-type stock were then plated as dense suspensions on Vogel's acetate and observed for vigorous growth after 24 h in an attempt to isolate further mutants with similar phenotypes. This procedure yielded only one further potential mutant after screening 10^7 conidia. Comparative growth curves for these strains (Fig. 1) revealed that both grew faster than wild-type on acetate medium. In addition, the putative mutants appeared disadvantaged compared with the wild-type during growth on sucrose.

Genetic characterization

The primary mutations were initially re-isolated from a back-cross to wild-type, almost half of the progeny from which exhibited the accelerated acetate growth (Aag) phenotype as would be expected for a single mutation.

Fig. 1. Growth of wild-type (□), Aag-1-1 (T13) (Δ) and Aag-1-2 (T14) (○) from similar conidial inocula on equivalent amounts of sucrose (a) or acetate (b) in shaken cultures.

However, a number of these progeny failed to grow at all on acetate plates. In a further out-cross to *am*¹³² to generate double mutants for heterokaryon complementation tests, both fast-acetate-growth mutations gave rise to a few acetate non-utilizing progeny. Five of these were selected from each cross and tested for heterokaryotic complementation on liquid acetate medium with all the acetate non-utilizing classes available in the laboratory (*acu-1*, -3, -5, -6, -7, -8, -9, -10, -11, -12, -13 and *acp*⁶). All but one of the *acu* strains proved to be new alleles of *acu-5*, the structural gene for the primary acetate-activating enzyme acetyl-CoA synthetase. The single exception to this, designated *acu-14*, was able to complement all of the known mutations and was therefore considered to represent a new acetate non-utilizing class.

The *am* Aag double mutants re-isolated from the out-crosses were combined in heterokaryons with *trp-3* and *pyr-4* strains. Heterokaryons from both Aag strains with both auxotrophic partners demonstrated faster-than-wild-type growth on acetate plates, although perhaps not to the same degree as the initial mutants. It would appear that

both mutations have semi-dominant characteristics. Representative strains of both mutants of opposite mating type were then crossed and their progeny were tested for growth on acetate. Again a few of these turned out to be acetate non-utilizers; however, the rest retained the *Aag*⁺ phenotype. As there were no wild-type recombinants from this cross, the mutations were considered to be allelic representatives of a single gene (*Aag1-1* and *Aag1-2*). To demonstrate further that the mutations represent a common gene and to map the new locus, *Aag1-1* and *Aag1-2* were crossed to the triple translocation strain *alcov csp* (Perkins, 1964). The *alcov csp* strains bear the visible markers *albino* (white conidia), *cot-1* (temperature-sensitive colonial growth) and *ylo* (yellow conidia which are epistatic to white), which identify the six linkage groups involved in the interchanges, and *csp-2* (conidial separation), which identifies the remaining independent linkage group (LGVII). Over 100 ascospore progeny that were recovered from these crosses were scored for the visible markers and the fast acetate growth phenotype. Both crosses indicated loose linkage to *csp-2* on LGVII, although spore germination was notably low in these crosses (< 50%). Recombination frequencies with all the other markers did not differ significantly from 50%. To confirm the preliminary assignment of the *Aag-1* mutation to LGVII, the *Aag1-1* allele was crossed with a secondary linkage group tester, FGSC 4142, which carries the *nic-3*, *met-7* and *arg-10* markers. However, *Aag1-1* did not demonstrate significant linkage to the *nic-3* (44.6%), *met-7* (46.5%) and *arg-10* (48%) markers. *Aag-1* was therefore crossed independently with three further tester strains carrying markers representing the remaining linkage groups (*al-1 arg-5*, FGSC 1206; *cot-1 al-3*, FGSC 1244; *trp-1 ylo-1*, FGSC 1208). In these crosses, linkage was only observed with the *arg-5* marker (33.0%) located on LGII.

As the *Aag-1* mutations were repeatedly found to produce new alleles of *acu-5* in spore progeny and were found to map to the same linkage group as the *acu-5* gene, it is possible that the *Aag-1* isolates were either initially double mutants harbouring a closely linked functionally unstable allele of *acu-5* or were unstable alleles of *acu-5* itself conferring a pleiotropic phenotype. To investigate these possibilities, mutant *Aag1-1* was crossed to an independent allele of *acu-5* (J118) and the spore progeny were tested for growth on acetate medium. The cross yielded 12.9% (18/140) wild-type progeny, implying a recombination frequency of 26% as the *Aag1-1* mutants will be epistatic to *acu-5* (J118) in the reciprocal class. The *acu* class (68/140) were bolstered by a similar number (19) to the wild-type class over the fast-acetate-growth class (49/140). These data confirm that the *Aag1-1* and the *acu-5* genes are on the same linkage group but are not allelic nor are they particularly closely linked.

Biochemical properties

Initially mycelial extracts of the *Aag-1* mutations prepared following growth on sucrose (non-induced) and subsequent growth on acetate (induced) were assayed for the enzymes of the glyoxylate cycle, gluconeogenesis and

Table 1. Enzyme activities of wild-type *N. crassa* and the *Aag-1* mutants on various carbon sources

Specific activities were measured in cell-free extracts after 16 h growth on sucrose medium (non-induced) and following a subsequent 8 h incubation in acetate, ethanol, quinate, cellulose or xylan (induced). Values represent the means of at least two sets of duplicate assays. NT, Not tested.

Strain	Enzyme activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) on:					
	Sucrose	Acetate	Ethanol	Quinate	Cellulose	Xylan
Acetyl-CoA synthetase						
ST.A	0.132	0.463	0.283	0.282	0.122	NT
T13	0.526	0.914	0.316	0.437	0.114	NT
T14	0.476	0.997	0.383	0.402	0.146	NT
Isocitrate lyase						
ST.A	0.35	2.81	0.92	0.76	0.22	0.21
T13	0.91	12.83	3.83	1.83	0.85	0.81
T14	2.22	8.75	1.83	2.56	0.14	1.62
Malate synthase						
ST.A	0.31	1.82	1.02	1.42	NT	NT
T13	0.88	3.13	2.33	2.11	NT	NT
T14	0.95	2.37	2.19	1.98	NT	NT
Phosphoenolpyruvate carboxykinase						
ST.A	0.009	0.039	0.019	0.021	0.001	0.001
T13	0.006	0.032	0.021	0.023	0.006	0.004
T14	0.011	0.029	0.018	0.022	0.001	0.001
Fructose-1,6-biphosphatase						
ST.A	0.28	0.35	0.33	0.38	0.11	0.16
T13	0.25	0.39	0.29	0.35	0.13	0.18
T14	0.28	0.35	0.31	0.36	0.21	0.21
Alcohol dehydrogenase						
ST.A	0.054	0.068	0.225	0.057	0.074	0.052
T13	0.053	0.052	0.305	0.062	0.111	0.139
T14	0.063	0.071	0.266	0.061	0.055	0.058

acetate mobilization (Table 1). It is apparent that the primary acetate-utilizing enzymes (acetyl-CoA synthetase, isocitrate lyase and malate synthase) were all elevated compared to the wild-type under non-inducing conditions. To examine whether the observed derepression was unique to the acetate assimilatory enzymes or was characteristic of other carbon catabolic pathways, a number of catabolic enzyme levels were measured under repressed and derepressed conditions. It is clear from these measurements (Table 1) that none of the other pathways examined are subject to derepression in the *Aag-1* mutants. As the repressed levels of acetyl-CoA synthetase were abnormally high, it seemed likely that the *Aag-1* mutants would show increased sensitivity to the antimetabolite fluoroacetate. Fluoroacetate undergoes lethal synthesis via fluoroacetyl-CoA to fluorocitrate and poisons aconitase. The *Aag-1* mutants were indeed only tolerant up to 0.20 M whereas the wild-type could tolerate 0.35 M fluoroacetate.

To ascertain whether the observed derepressed levels of

Fig. 2. Northern blot analysis of wild-type and the *Aag-1-1* (T13) and *Aag-1-2* (T14) alleles. Total RNAs were isolated after 16 h growth on sucrose (S) or following 6 h induction on acetate (A). Independent Northern transfers were probed with (a) 6 kb *Hind*III fragment of the *acu-5* acetyl-CoA synthetase gene, (b) 1.7 kb *Bam*HI fragment of the *acu-9* malate synthase gene, (c) 1.2 kb *Hind*III fragment of the *acu-3* gene and (d) 3.1 kb *Hind*III fragment of the *bml* β -tubulin gene as a constitutive mRNA control. Aliquots (approx. 20 μ g) of the RNA preparations were initially probed for β -tubulin and the RNA quantities were standardized in repeated blots using a laser densitometer according to the observed signal. The boxed figures at the bottom of each part represent the signal strength in pixels.

the acetate-responsive enzymes were due to the release of otherwise inactive enzymes (translational control) or due to inopportune mRNA synthesis (transcriptional control). Northern transfers were undertaken. Fig. 2 shows the relative mRNA levels for the wild-type and *Aag-1* mutant strains after mycelial growth on sucrose and acetate of the acetate responsive structural genes for acetyl-CoA synthetase (*acu-5*), isocitrate lyase (*acu-3*) and malate synthase (*acu-9*). It is clear that compared with the wild-type both of the *Aag-1* alleles demonstrate marked transcriptional derepression on sucrose.

DISCUSSION

The initial fortuitous selection of a spontaneous mutant of *N. crassa* able to produce fast vigorous growth on acetate plates provided the basis for the selection of potentially novel mutants. Subsequent selection for further mutants of this class, leading to one further mutant which proved to be allelic, demonstrated the mutation to be rare. Indicative of their potential as regulatory mutants, both *Aag-1* alleles gave rise to the semi-dominant phenotype of faster-than-wild-type growth on acetate as sole carbon source. However, both had the physiological disadvantage of slower-than-wild-type growth on the preferred carbon source, sucrose. The *Aag-1* mutants demonstrated the specific derepression of the acetate inducible enzymes acetyl-CoA synthetase, isocitrate lyase and malate synthase on the normally repressing carbon source, sucrose. Enzymes in other carbon-assimilatory pathways were unaffected, as were the gluconeogenic enzymes co-induced by acetate, phosphoenolpyruvate carboxykinase and fructose-1,6-biphosphatase. A simple physiological explanation of the growth phenotype appears to be that the standing levels of the acetate-assimilatory enzymes enable the mutants to utilize the new carbon source quicker than the wild-type. Following transfer to acetate the mutants also demonstrate enzyme induction to levels greater than in the wild-type. Acetyl-CoA synthetase levels are normally carefully regulated in the cell because excess cytosolic acetyl-CoA can be toxic. Acetyl-CoA synthetase levels are regulated not only under conditions of catabolite repression but also following induction, as the enzyme and corresponding mRNA levels do not approach those observed for the co-inducible glyoxylate cycle enzymes. The cytosolic acetyl-CoA concentration is further modulated by a second co-inducible enzyme, acetyl-CoA hydrolase (Connerton *et al.*, 1992), to prevent possible toxic accumulation. The levels of acetyl-CoA synthetase in the *Aag-1* mutants are therefore a potential source of physiological stress. To relieve this stress the *Aag-1* mutation must either revert or force a second site mutation. When growth on acetate is not selected, a prime candidate for this is the structural gene for acetyl-CoA synthetase. The observation that a variable quotient of homokaryotic ascospore progeny are *acu-5* alleles may reflect this.

Messenger RNA levels of the acetate-responsive genes, detected on Northern transfers, demonstrated the abnormal derepression of these genes during growth on sucrose. The derepression is clearly due to a loss of

transcriptional control. Two types of mutational event may directly cause transcriptional derepression: (i) a mutation leading to the loss or dysfunction of a repressor protein, or (ii) a missense mutation in an activator protein such that it can no longer be recognized directly by its cognate repressor or it can no longer be displaced from its target promoter binding site. As the *Aag-1* mutants are semi-dominant, (i) is not compatible and can therefore be discounted. A mutation in the putative activator would have a dominant phenotype.

ACKNOWLEDGEMENTS

P. T. C. was the recipient of an overseas scholarship from the Indian Government.

REFERENCES

- Apirion, D. (1965). The two way selection of mutants and revertants in respect of acetate utilization and resistance to fluoroacetate in *Aspergillus nidulans*. *Genet Res* **6**, 317–329.
- Armitt, S., McCullough, W. & Roberts, C. R. (1976). Analysis of acetate non-utilizing (*acu*) mutants in *Aspergillus nidulans*. *J Gen Microbiol* **92**, 263–282.
- Barth, G. (1985). Genetic regulation of isocitrate lyase in the yeast *Yarrowia lipolytica*. *Curr Genet* **10**, 119–124.
- Beever, R. E. (1975). Regulation of 2-phosphoenolpyruvate kinase and isocitrate lyase in *Neurospora crassa*. *J Gen Microbiol* **86**, 197–200.
- Casselton, L. A. & Casselton, P. J. (1974). Functional aspects of fluoroacetate resistance in *Coprinus* with special reference to acetyl-CoA synthetase deficiency. *Mol & Gen Genet* **132**, 255–264.
- Connerton, I. F. (1990). Premiotic disruption of the *Neurospora crassa* malate synthase gene by native and divergent DNAs. *Mol & Gen Genet* **223**, 319–323.
- Connerton, I. F., Fincham, J. R. S., Sandeman, R. A. & Hynes, M. J. (1990). Comparison and cross-species expression of the acetyl-CoA synthetase genes of the ascomycete fungi, *Aspergillus nidulans* and *Neurospora crassa*. *Mol Microbiol* **4**, 451–460.
- Connerton, I. F., McCullough, W. & Fincham, J. R. S. (1992). An acetate-sensitive mutant of *Neurospora crassa* deficient in acetyl-CoA hydrolase. *J Gen Microbiol* **138**, 1797–1800.
- Dixon, G. H. & Kornberg, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochem J* **72**, 3P.
- Flavell, R. B. & Fincham, J. R. S. (1968a). Acetate non-utilizing mutants of *Neurospora crassa*. 1. Mutant isolation, complementation studies and linkage relationships. *J Bacteriol* **95**, 1056–1062.
- Flavell, R. B. & Fincham, J. R. S. (1968b). Acetate non-utilizing mutants of *Neurospora crassa*. 2. Biochemical deficiencies and roles of certain enzymes. *J Bacteriol* **95**, 1063–1068.
- Katz, M. E. & Hynes, M. J. (1989). Isolation and analysis of the acetate regulatory gene *facB*, from *Aspergillus nidulans*. *Mol Cell Biol* **12**, 5696–5701.
- Kinnaird, J. H., Keighen, M. A., Kinsey, J. A. & Fincham, J. R. S. (1982). Cloning of the *am* (glutamate dehydrogenase) gene of *Neurospora crassa* through the use of a synthetic DNA probe. *Gene* **20**, 387–396.
- Kornberg, H. L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem J* **99**, 1–11.
- Kujau, M., Weber, H. & Barth, G. (1992). Characterization of mutants of the yeast *Yarrowia lipolytica* deficient in acetyl-coenzyme A synthetase. *Yeast* **8**, 193–203.
- McCullough, W. & Roberts, C. F. (1980). The genetic control of isocitrate lyase in *Aspergillus nidulans*. *FEBS Lett* **41**, 238–242.
- McCullough, W. & Shanks, A. (1993). Properties of genes involved in the control of isocitrate lyase production in *Aspergillus nidulans*. *J Gen Microbiol* **139**, 509–511.
- Maconochie, M. K., Connerton, I. F. & Casselton, L. A. (1992). The *acu-1* gene of *Coprinus cinereus* is a regulatory gene required for induction of acetate utilisation enzymes. *Mol & Gen Genet* **234**, 211–216.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marathe, S., Connerton, I. F. & Fincham, J. R. S. (1990). Duplication-induced mutation of a new *Neurospora* gene required for acetate metabolism: properties of the mutant and predicted amino acid sequences of the protein product. *Mol Cell Biol* **10**, 2638–2644.
- Owen, N. E., Chaure, P. T. & Connerton, I. F. (1992). Isolation and characterization of new fluoroacetate/acetate non-utilizing mutants of *Neurospora crassa*. *J Gen Microbiol* **138**, 2599–2608.
- Perkins, D. D. (1964). Multiple interchange stocks for linkage detection. *Neurospora Newsl* **6**, 22–23.
- Rao, T. K. & Debusk, A. G. (1977). An inducible acetate transport system in *Neurospora crassa* conidia. *Biochim Biophys Acta* **470**, 475–483.
- Selker, E. U., Cambereri, E. B., Jensen, B. C. & Haack, K. R. (1987). Rearrangement of duplicated DNA in specialized cells of *Neurospora*. *Cell* **51**, 741–752.
- Sokolovsky, V., Kaldenhoff, R., Ricci, M. & Russo, V. E. A. (1990). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. *Fungal Genet Newsl* **37**, 41–43.
- Tatum, E. L., Barratt, R. W. & Cutter, V. M. (1949). Chemical induction of colonial paramorphs in *Neurospora* and *Syncephalastrum*. *Science* **109**, 509–511.
- Thomas, G. H., Connerton, I. F. & Fincham, J. R. S. (1988). Molecular cloning, identification and transcriptional analysis of genes involved in acetate utilization in *Neurospora crassa*. *Mol Microbiol* **2**, 599–606.
- Vogel, H. J. (1956). A convenient growth medium for *Neurospora* (Medium N). *Microb Genet Bull* **13**, 42–43.
- Westergaard, M. & Mitchell, H. K. (1947). A synthetic medium favouring sexual reproduction. *Am J Bot* **34**, 573–577.

Recived 11 October 1994; accepted 20 February 1995.