Isolation and characterization of new fluoroacetate resistant/acetate non-utilizing mutants of *Neurospora crassa*

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Sixty-two mutants of the filamentous fungus *Neurospora crassa* were isolated on the basis of resistance to the antimetabolite fluoroacetate. Of these, 14 were unable to use acetate as sole carbon source (acetate non-utilizers, *acu*) and were the subject of further genetic and biochemical analysis. These mutants fell into four complementation groups, three of which did not complement any known *acu* mutants. Mutants of complementation group 3 failed to complement *acu-8*, demonstrated similar phenotypic properties and proved to be closely linked (less than 2% recombination) but not allelic. Representatives of groups 2 and 4 were mapped to independent loci; the single representative of group 1 could not be mapped. The four complementation groups were therefore designated as genes *acu-10* to *acu-13* respectively. All the mutants demonstrated normal acetate-induced expression of acetyl-CoA synthetase and the unique enzymes of the glyoxylate cycle and gluconeogenesis. The nature of these mutations is therefore quite different to those reported for other fungal species. Mutant *acu-11* was unable to fix labelled acetate, indicating the loss of an initial transport function; partial enzyme lesions were observed for *acu-12* (acetyl-CoA hydrolase) and *acu-13* (acetate-inducible NAD⁺-specific malate dehydrogenase).

Introduction

Many fungi can utilize acetate as sole carbon source. 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), together with the basidiomycete *Coprinus cinereus* (Casselton & Casselton, 1974) and the yeast *Yarrowia lipolytica* (Barth, 1985).

Acetate is incorporated via acetyl-CoA into the glyoxylate cycle, an anaplerotic or topping-up pathway to the TCA cycle. This involves two reactions, catalysed by the enzymes isocitrate lyase and malate synthase, which bypass the two decarboxylation steps of the TCA cycle. In addition to the enzymes of the glyoxylate cycle, three more enzymes play critical roles in acetate metabolism: phosphoenolpyruvate carboxykinase catalyses the conversion of oxalacetate to phosphoenolpyruvate, which links acetate metabolism to gluconeogenesis;

malic enzyme, which is necessary for the conversion of malate to free pyruvate as an essential precursor of amino acid synthesis; and fructose-1,6-bisphosphatase, to provide a supply of hexose sugars. The enzyme NADP⁺-specific isocitrate dehydrogenase also has an effect on the glyoxylate cycle enzymes by competing for isocitrate and may, therefore, affect the flow of metabolites through the cycle. NAD⁺- and NADP⁺-specific malate dehydrogenases, together with glutamate oxalacetate transaminase may enable the flow of NAD⁺ and NADP⁺ between the glyoxysome and mitochondrion (Cioni *et al.*, 1978).

The first mutants in the filamentous fungi with an inability to utilize acetate were those isolated on the basis of fluoroacetate resistance in *Aspergillus nidulans*, mapping at three distinct loci: *facA*, deficient in acetyl-CoA synthetase *facB*, the regulatory mutation, and *facC*, of unknown function (Apirion, 1965). The *facB* mutations resulted in pleiotropic enzyme deficiencies in glyoxylate cycle enzymes when induced on acetate (Apirion, 1965; Armstrong *et al.*, 1976). Two classes of phenotypic mutant were identified by Apirion (1965): *fac* (*fac*⁺ *acu*) and *fan* (*fac*⁺ *acu*⁺); the first of these has been the subject of further study. The rationale behind this selection was that fluoroacetate is converted to mitochondrial fluoro-

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Abbreviations: DTNB, 5',5'-dithiobis(2-nitro)benzoic acid; RIP, repeat induced point (mutation).

citrate by acetyl-CoA synthetase and citrate synthase. Monofluorocitrate is a potent inhibitor of aconitase (Peters, 1957) and citryl-glutathione synthetase (Kun *et al.*, 1977); therefore mutants that do not mobilize the fluoroacetate will be resistant.

Armstrong *et al.* (1976) identified ten new acetate non-utilizing (*acu*) loci, in addition to the fluoroacetate resistant mutants (Armstrong *et al.*, 1976). The *acuA* mutants were allelic to *facA* and deficient in acetyl-CoA synthetase. Mutants were also identified in the structural genes for isocitrate lyase (*acuD*), malate synthase (*acuE*), phosphoenolpyruvate carboxykinase (*acuF*), fructose-1,6-bisphosphatase (*acuG*) and malic enzyme (*acuK* and *acuM*); however, none were equivalent to *facB*. These loci were not linked and occurred scattered throughout the genome.

Flavell & Fincham (1968*a*) isolated numerous mutants of *Neurospora crassa* on the basis of their loss of ability to use acetate as sole carbon source. They fell into seven distinct complementation groups mapping to seven independent loci. Three of these groups possessed single enzyme deficiencies similar to those found in *Aspergillus*: *acu-3* and *acu-5* lacked isocitrate lyase and acetyl-CoA synthetase respectively, whereas *acu-6* was deficient in phosphoenolpyruvate carboxykinase. Later, Rao & Debussk (1977) reported an acetate non-utilizing permease deficient mutant and designated it *acp⁻ⁱ*.

Thomas *et al.* (1988) endeavoured to clone genes expressed during the adaptation of *Neurospora* from sucrose to acetate as sole carbon source. Two 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 generate a novel *acu* mutant, *acu-8*, utilizing the RIP (repeat induced point mutation) phenomenon of *Neurospora* (Marathe *et al.*, 1990), and now thought 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). RIP is a premeiotic phenomenon observed in *N. crassa* whereby duplicated sequences undergo extensive cytosine methylation and numerous GC→AT base transitions, thereby disrupting the participating sequences (Selker *et al.*, 1987; Cambareri *et al.*, 1989).

The current paper describes the isolation and characterization of a further four acetate non-utilizing mutants that were selected initially on the basis of fluoroacetate resistance.

Methods

Strains. The strains used in biochemical and heterokaryon tests were wild-type 74 A/a (STA/a); *acp⁻ⁱ* (Rao & Debust, 1977); *acu-1* (JI48); *acu-3* (JI38); *acu-5* (JI18; 94-78); *acu-6* (JI31); *acu-7* (JI36) (Flavell &

Fincham, 1968*a b*); *acu-8* (T27-1-8) (Marathe *et al.*, 1990); and *acu-9* (T11-1) (Connerton, 1990). Linkage group marker strains used for mapping gene loci were *alcoy* [*T(I;II)al-1*; *T(III;V)tylo-1*; *T(IV;V)cor*] (Perkins, 1964); *arg-3*; *his-3*; *nic-2*; *al-1* (FGS5544) I; *trp-3* (FGS4093) IIR; *arg-10* (FGS4141) VII.

Media. Vogel's sucrose minimal medium contained 1% (w/v) Vogel's salts (Vogel, 1956) supplemented with d-biotin (1 mg l⁻¹) with 1.5% (w/v) sucrose (BDH) as carbon source. Solid medium contained 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). Vogel's acetate minimal medium contained sodium acetate (BDH) as sole carbon source, routinely at a final concentration of 40 mM (optimal for wild-type growth). Other carbon sources are detailed in the text, for which solid medium included 0.2% sorbose. Vogel's fluoroacetate medium contained sodium fluoroacetate (Aldrich/Sigma) at a final concentration of 500 mM supplemented with 0.5% glucose and fructose and 0.2% sorbose. All solid media contained 1.5% agar (Difco). Concentrations of other supplements were as follows: ethanol 0.5% (w/v); butyrate, glycerol and oleate, all 1% (w/v); L-arginine, -histidine, -methionine, -tryptophan, all 1 mM; nicotinamide, 2 µg ml⁻¹.

Mutagenesis. A 5 ml suspension of 10⁷–10⁸ viable conidia ml⁻¹ (STA) was treated with 200 µl of a 1 mg ml⁻¹ solution of NTG (*N*-methyl-*N*-nitrosoguanidine, Sigma) and gently shaken at room temperature for 20 min to give 90% killing. The suspension was washed three times with 10 ml of sterile water and resuspended in 5 ml of sterile water; 200 µl of suspension was then plated out directly onto fluoroacetate, and overlaid with 10 ml of fresh fluoroacetate medium after 3 h incubation at 30 °C before continued incubation for 3–5 d.

Growth tests. A small quantity of conidia was streaked onto plates containing the appropriate growth medium including sorbose and growth was scored (as +/–) after 3 d incubation at 30 °C. Growth tests on medium without sorbose were scored after 16 h incubation at 30 °C, except when scoring *cor* for which 37 °C was used. For scoring in liquid medium, 0.05 ml of a conidial suspension was used to inoculate 2 ml of medium and growth scored after 2–3 d.

Crossing procedure and revival of ascospores. Sexual crosses were made on 1 × Westergaard and Mitchell's crossing medium (Westergaard & Mitchell, 1947), whether on slants with 1.5% sucrose or in Petri dishes containing discs of 3 MM filter paper (cellulose) as carbon source to increase the fertility of crosses. Ascospores were collected from the glass or plastic surfaces after release and germinated on Vogel's sucrose medium plus the appropriate supplements after heatshock at 60 °C.

Enzyme assays. Gluconeogenic, glyoxylate cycle and acetate mobilizing enzyme were assayed together with those enzymes of the TCA cycle potentially involved in acetate utilization from wild-type and representative mutant strains. Mycelial pads were grown in Vogel's sucrose medium for 22 h, followed by transfer to acetate medium for 6 h at 30 °C with shaking. Crude enzyme extracts were then prepared by grinding the mycelial pads with chilled 0.1 M-sodium phosphate buffer (pH 7.2) and acid washed sand. Supernatants were retrieved after centrifugation at 6000 g for 3 min in a microcentrifuge and stored on ice for use in enzyme assays and protein determinations.

Isocitrate lyase (EC 4.1.3.1). Assayed as described by Dixon & Kornberg (1959). The assay mixture contained 0.1 M-sodium phosphate buffer, pH 7.2, 10 mM-MgCl₂ and the reaction was initiated by addition of 12.5 mM-DL-isocitric acid at 30 °C. The reaction was monitored at 324 nm for conversion of 0.1 M-phenylhydrazine. HCl to glyoxylatephenylhydrazone.

Acetyl-CoA synthetase (EC 6.2.1.1). Assayed as described by Jones & Lipman (1959) with modifications available from Sigma, coupling hydroxylamine to acetyl-CoA formation. The colour reaction was

developed using acid ferric chloride and measured at 546 nm against a reagent blank.

Malate synthase (EC 4.1.3.2). Assayed as described by Dixon & Kornberg (1959). The reaction mixture contained 0.1 M-sodium phosphate buffer, 10 mM-MgCl₂, 0.02 mM-acetyl-CoA and 0.1 ml of enzyme extract, and the reaction was initiated by addition of 5 mM-glyoxylate after an initial 3 min incubation at 30 °C; the disappearance of acetyl-CoA was monitored at 232 nm. Alternatively, the reaction was stopped by addition of 0.5 µmol 5,5'-dithiobis(2-nitro)benzoic acid (DTNB), which reacts with free CoA-SH. The increase in absorbance of the yellow product was measured at 412 nm.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32). The reaction mixture contained 0.1 M-Tris/HCl, pH 7.2, 10 mM-MgCl₂, 25 mM-KHCO₃, 1 mM-ADP, 2.5 mM-NADH, 15 mM-phosphoenolpyruvate and 0.05 ml of enzyme extract. The reaction was initiated by addition of phosphoenolpyruvate after 2–3 min incubation at 30 °C and the oxidation of NADH was monitored at 340 nm in the presence of excess endogenous malate dehydrogenase.

NADP⁺-specific malic enzyme (EC 1.1.1.40). The reaction mixture contained 0.75 ml 0.1 M-Tris/HCl, pH 7.2, 0.1 ml 0.1 M-MgCl₂, 0.05 ml 1 mM-dithiothreitol (DTT), 0.01 ml 25 mM-NADP⁺, 0.05 ml 1 M-malate and 0.05 ml of enzyme extract in 1 mM-DTT. The formation of pyruvate was followed after 5 min reaction time by colour development with 0.5 ml 0.1% 2,4-dinitrophenylhydrazine in 2 M-HCl.

Glutamate:oxaloacetate transaminase (EC 2.6.1.15). The reaction mixture contained 0.8 ml 300 mM-sodium phosphate buffer, pH 7.4, 0.05 ml 5 mM-NADH, 0.1 ml 180 mM-2-oxoglutarate and 0.05 ml of enzyme extract. The reaction was initiated by addition of aspartate at 30 °C and the disappearance of NADH was measured at 340 nm.

NAD⁺-specific malate dehydrogenase (EC 1.1.1.37). The reaction mixture contained 0.84 ml 300 mM-sodium phosphate buffer, pH 7.4, 0.05 ml 5 mM-NADH, 0.01 ml 250 mM-oxaloacetate and 0.05 ml of diluted enzyme extract. Due to the excess of NAD⁺-specific malate dehydrogenase in the extract, it was necessary to dilute the extract 1/50 in phosphate buffer. The reaction was initiated by addition of oxaloacetate at 30 °C. The disappearance of NADH was measured at 340 nm. Activity gels were run using 10% (w/v) polyacrylamide gels in a continuous buffer system containing 0.05 M-Tris/0.38 M-glycine, pH 8.9, with cell-free extracts. Isoenzymes were stained using 0.05 M-Tris/HCl, pH 8.9, 0.16% NAD⁺, 0.0016% phenazine ethosulphate, 0.045% nitroterazolum blue and 0.2 M-DL-malate for 20 min in the dark.

NADP⁺-specific malate dehydrogenase (EC 1.1.1.82). The reaction mixture contained 0.99 ml 300 mM-sodium phosphate buffer, pH 7.4, 0.01 ml 25 mM-NADP⁺, 0.05 ml 1 M-malate and 0.05 ml of enzyme extract. The reaction was initiated by addition of malate at 30 °C. The formation of NADPH was measured at 340 nm.

NAD⁺-specific isocitrate dehydrogenase (EC 1.1.1.41). The reaction mixture consisted of 0.83 ml of 250 mM-sodium phosphate buffer, pH 7.2, 0.01 ml 25 mM-NAD⁺, 0.1 ml 0.1 M-MgCl₂, 0.05 ml 500 mM-DL-isocitrate and 0.01 ml of enzyme extract. The appearance of NADH was followed at 340 nm.

NADP⁺-specific isocitrate dehydrogenase (EC 1.1.1.42). The reaction mixture was essentially similar to that for NAD⁺-specific isocitrate dehydrogenase except that 25 mM-NADP⁺ replaced NAD⁺ and the reaction was followed as the appearance of NADPH.

Acetyl-CoA hydrolase (EC 3.1.2.1). Acetyl-CoA hydrolase was assayed in post-organellar fractions (extracted in 0.4 M-sucrose, 10 mM-Tricine, pH 7.2) by measuring the appearance of free CoA-SH using DTNB (Connerton *et al.*, 1992). The assay mixture contained 0.8 ml 50 mM-Tris/HCl, pH 7.5, 0.1 ml 2 mM-acetyl-CoA and 0.1 ml of

and was incubated at 30 °C for 10 min before termination and colour development with 0.5 µmol DTNB.

[¹⁴C]Acetate uptake. A suspension of 10⁶ conidia in 25 ml of Vogel's minimal medium containing 40 mM-[¹⁴C]acetate [0.2 µCi ml⁻¹ (7.4 kbq ml⁻¹)] was incubated at 30 °C, stirring gently and shaking; samples (2 ml) were removed at 2 min intervals for 16 min. These samples were washed on a Millipore membrane filter (pore size 22 µm) with 10 ml of Vogel's minimal medium before drying at 65 °C for 1 h and scintillation counting against known standards.

Estimation of protein concentration. The concentration of protein in the enzyme extracts was determined using the Coomassie blue G-250 based protein assay reagent (Pierce). 'Ready to use' reagent (200 µl) was added to a well in a 96-well microtitre tray together with 5 µl bovine serum albumin (Sigma) ranging from 0.25–1.5 mg ml⁻¹. A suitable dilution of enzyme extract (5 µl) was added in separate wells. The protein concentration was determined at 720 nm (filter 7) using the Titertek Multiskan MCC system.

Results

Selection and isolation of mutants

A concentration of sodium fluoroacetate that inhibited growth of the wild-type strains (STA/a), but supported growth of *acu-8* (Marathe *et al.*, 1990), which was found to be fluoroacetate resistant, was determined in preliminary plate assays. A sandwich technique similar to that adopted by Apirion (1965) was used in the selection procedure. Conidial suspension (100–200 µl) at approximately 10⁶ viable conidia ml⁻¹ was plated directly onto various concentrations of Vogel's fluoroacetate medium. After 3–4 h incubation at 30 °C, the germinating spores were overlaid with fresh medium. Resistant colonies appeared after 3–4 d. *Neurospora* proved to be surprisingly tolerant to fluoroacetate: growth of STA/a was inhibited completely only at 0.4 M, whereas *acu-8* continued to grow well up to 0.5 M-fluoroacetate.

Of 10⁶ conidia screened in a series of isolation experiments, 62 vigorously growing fluoroacetate resistant colonies were selected; of these, eight were initially discarded due to poor growth on transfer to Vogel's sucrose medium. Conidial stocks from these were scored for growth on solid Vogel's acetate medium. Fourteen isolates were acetate non-utilizing mutants (*fac⁺ acu*), the majority (*fac⁺ acu⁺*) fell into the *fan* class described by Apirion (1965). This second class was not examined further.

Genetic analysis of the *fac⁺ acu* mutants

On the basis of growth tests with heterokaryotic conidia on liquid acetate medium, the mutants tested were arranged into four provisional complementation groups (Fig. 1). Each of these was considered to contain alleles of a single gene. No allelic complementation was

Fig. 1. Complementation analysis of *fac*⁺ *acu* mutants. The mutants have been arranged into four groups on the basis of their ability to complement each other in heterokaryons. Numbers 1–14 were isolated as S10-3, S30-12, S40-4, S10-4, S10-6, S10-11, S10-14, S10-2, S10-8, S10-15, S10-16, S20-1, S30-2 and S30-9 respectively. ●, Complementation; ○, no complementation.

observed. A linear complementation matrix was also performed with the known *acu* mutants. Apart from S10-4 (phenotypically the tightest representative of complementation group 3), which showed no complementation with *acu*-8, the three remaining groups complemented all available *acu* mutants.

Representatives from each complementation group exhibiting tight *Acu*[−] phenotypes were back-crossed twice to wild-type of the opposite mating type (STA) to ensure that the *Acu*[−] phenotype was not due to mutation at multiple loci. Twenty ascospores from each cross to the wild-type strain were screened for their ability to utilize acetate. The ratio of *acu*⁺/*acu* was approximately 1:1 for three out of the four representatives. However, a chance observation on the growth characteristics of the mutant S10-3 after crossing to the wild-type (Fig. 1, complementation group 1) revealed a possible double mutant. The absolute ratio of *acu*⁺/*acu*, as opposed to those ascospore progeny which grew weakly on acetate, from random ascospores was approximately 3:1 (27:13), implying that only the double mutant expressed the stringent phenotype. Further growth analysis revealed the second mutation to be unstable as four strains of the phenotype *Acu*⁺ *Alc*[−] (alcohol) were identified within the acetate utilizing ascospore progeny (27). The weak *Acu*[−] phenotype was subsequently re-isolated from a third backcross to be mapped. Cultures re-isolated from backcrosses to wild-type that continued to exhibit a tight *Acu*[−] phenotype were crossed to the triple translocated strain *alcoy* (Perkins, 1964), bearing the visible markers *albino* (white conidia), *cot-1* (temperature-sensitive colonial growth) and *ylo* (yellow conidia which are epistatic

to white). Representative strains S30-12 (group 2), S10-4 (group 3) and S30-9 (group 4) yielded abundant ascospore progeny from which 100 random ascospores were picked onto Vogel's sucrose minimal medium and scored for their visible markers and acetate utilization. However, the single representative of complementation group 1 (S10-3) did not produce a fertile cross with any of its derivative *acu* progeny. It is possible that this strain, which was originally isolated in conjunction with a second mutation, may have undergone some rearrangement and it remains unmapped.

Clear linkage was observed for S30-9 with albino indicating either linkage group I or II. Linkage to LGII was confirmed by crossing to the *trp-3* marker of LGII (22.7%) and *albino* of LGI (linkage data detailed in Table 1). No linkage was apparent for S30-12 or S10-4, which indicated preliminary assignment to linkage group VII, the only linkage group not involved in the interchange present in *alcoy*. Strains of S30-12 and S10-4 were then crossed to *arg-10* on LGVII. S30-12 demonstrated linkage to *arg-10* (21%). However, S10-4 (complementation group 3), which initially showed no complementation with *acu*-8, demonstrated no linkage to *arg-10* on LGVII. As *acu*-8 was mapped to the distal end of the right arm of linkage group II (Marathe *et al.*, 1990), and if S10-4 was allelic, it would not necessarily have shown linkage to *alcoy*. S10-4 was subsequently crossed to *trp-3* on linkage group II to determine linkage and was found to be linked to *trp-3* (24%), but not as closely as *acu*-8 (7.8%). Crossing of S10-4 to *acu*-8 resulted in only four *acu*⁺ progeny of 405 tested, implying the mutations are not allelic but closely linked (<2%). To undertake these crosses was laborious as the strains did not prove to be a particularly fertile combination. This was the case regardless of which mating-type was employed or which mutant constituted the female parent. Whether due to the interaction of their phenotypes or to the genetic mishaps that have befallen them, these mutants, in addition to producing fairly infertile crosses, also suffered from low ascospore viability. These phenomena had been noted earlier with the *acu* mutants of Flavell & Fincham (1968a), although these problems were not encountered with *Aspergillus* (Armstrong *et al.*, 1976).

Based upon these relationships the new mutant classes were designated *acu*-10 (S10-3 complementation group 1), *acu*-11 (S30-12 and S40-4 of complementation group 2), *acu*-12 (S10-4 and the rest of complementation group 3) and *acu*-13 (S30-9 and the rest of complementation group 4).

Growth characteristics of the *fac* mutants

The *fac* mutants were tested for their growth responses on various carbon sources in liquid medium, except for

Table 1. Linkage group analysis of progeny from crosses between *fac*⁺ *acu* mutants and known genetic markers

Cross	Linkage group of marker	Progeny genotypes	No. observed	Conclusions (percentage recombination between <i>fac</i> ⁺ <i>acu</i> locus and marker)
S30-9 × <i>trp-3</i>	IIR	<i>acu</i> + + <i>trp-3</i> + + <i>acu trp-3</i>	40 11 14 1	S30-9 <i>acu</i> linked to <i>trp-3</i> (22.7%)
S30-9 × <i>al-1</i>	I	<i>acu</i> + + <i>al-1</i> + + <i>acu al-1</i>	35 26 42 18	S30-9 <i>acu</i> and <i>al</i> not linked (49.6%)
S10-4 × <i>arg-10</i>	VIIR	<i>acu</i> + + <i>arg-10</i> + + <i>acu arg-10</i>	19 24 22 17	S10-4 <i>acu</i> and <i>arg-10</i> not linked (47.5%)
S10-4 × <i>trp-3</i>	IIR	<i>acu</i> + + <i>trp-3</i> + + <i>acu trp-3</i>	31 19 9 7	S10-4 <i>acu</i> linked to <i>trp-3</i> (24%)
S30-12 × <i>arg-10</i>	VIIR	<i>acu</i> + + <i>arg-10</i> + + <i>acu arg-10</i>	28 20 10 3	S30-12 <i>acu</i> linked to <i>arg-10</i> (21%)

Table 2. Growth characteristics of *fac* mutants in *Neurospora crassa*

Growth responses of the mutants on various carbon sources are shown in comparison with growth of wild-type on hexose (+ + +). No growth is indicated by −, and increasingly better growth as (+), +, ++, +++. NT, Not tested.

Strain	Growth on:								Fluoroacetate (0.5 M) + glucose + fructose (0.5%)
	Glucose + fructose (0.5%)	Acetate (10 mM)	Acetate (40 mM)	Ethanol (0.5%)	Butyrate (1%)	Glycerol (1%)	Glutamate (1%)	Oleate (1%)	
STa	+++	+++	+++	+++	++	++	++	+++	−
<i>acp</i> [−]	+++	−	−	−	−	++	++	++	−
<i>acu-3</i>	+++	−	−	−	−	++	++	−	−
<i>acu-5</i>	+++	−	−	−	+	++	++	(+)	−
<i>acu-8</i>	+++	++	−	++	−	++	++	++	++
<i>acu-10</i>									
S10-3	+++	+	(+)	−	−	++	++	++	++
<i>acu-11</i>									
S30-12	+++	−	−	+	−	++	++	++	++
S40-4	++	(+)	−	(+)	NT	NT	NT	++	++
<i>acu-12</i>									
S10-4	+++	++	−	++	−	++	++	++	++
S10-6	+	+	(+)	+	NT	NT	NT	NT	++
S10-11	+	+	(+)	+	NT	NT	NT	NT	++
S10-14	++	+	(+)	++	NT	NT	NT	NT	++
<i>acu-13</i>									
S10-2	+++	+	−	−	−	++	++	++	++
S10-8	+++	+	−	−	NT	NT	NT	NT	++
S10-15	+++	+	−	−	NT	NT	NT	NT	++
S10-16	+	+	(+)	−	NT	NT	NT	NT	++
S20-1	++	+	(+)	−	NT	NT	NT	NT	++
S30-2	+++	+	−	−	NT	NT	NT	NT	++
S30-9	+++	+	−	−	−	++	++	++	++

fluoroacetate where solid agar plates were used (Table 2). Generally mutants within the same complementation group showed similar growth characteristics. Of the established *acu* mutants only *acu-8* demonstrated fluoroacetate resistance. Perhaps surprisingly the acetyl-CoA

synthetase deficient class (*acu-5*) were sensitive to fluoroacetate; in *Aspergillus nidulans* this is the most abundant fluoroacetate resistant class as these mutants fail to mobilize fluoroacetyl-CoA in lethal synthesis. Unusually, alleles of *acu-11* and *acu-12* showed growth on

ethanol unlike many other known *acu* mutants, the notable exception being *acu-8*. The mutants *acu-8* and *acu-12* (S10-4) have been shown to be closely linked and demonstrate quite similar properties: *acu-8* is fluoroacetate resistant like S10-4; *acu-8* does not complement S10-4 in heterokaryons; both have similar curious growth phenotypes, showing no growth on 40 mM-acetate, but growth on 10 mM-acetate and 0.5% ethanol (Table 2). At the standard pH of 5.2 for acetate medium, *acu-11* (S30-12) did not grow on either concentration of acetate. However, if the acetate medium was brought to pH 6.5, *acu-11* produced appreciable growth. The mutant was not generally acid sensitive, only in respect of growth on acetate.

None of the fluoroacetate resistant mutants were capable of growth on butyrate; the acetyl-CoA synthetase deficient class (*acu-5*) were the only acetate non-utilizers capable of this. Growth responses on oleate were also tested since oleate has been shown to induce the enzymes related to β -oxidation and has a profound influence on the ultrastructural content of cells inducing microbody formation (Kionka & Kunau, 1985). All the *fac*⁺ *acu* mutants showed growth on oleate; notably, only *acu-3* (deficient in isocitrate lyase) completely failed to grow. Presumably the glyoxylate cycle is required for growth on oleate but acetyl-CoA synthetase (*acu-5*) can be bypassed.

Biochemical properties of the *fac* mutant

Representatives from each complementation group were assayed for the presence of enzymes of the glyoxylate cycle, gluconeogenesis and those enabling acetate metabolite mobilization (Table 3). Despite lesions in acetyl-CoA synthetase leading to the majority of fluoroacetate resistant strains in other fungal species, all the mutants isolated in this study demonstrated inducible levels of the enzyme. Acetyl-CoA hydrolase is thought to modulate the cytosolic levels of acetyl-CoA, and a deficiency gives rise to the fluoroacetate resistant mutant *acu-8* (Connerton *et al.*, 1992). The enzyme was found to be inducible in all mutants. However, *acu-12* (S10-4 and other alleles not presented), which bears some similar phenotypic properties to *acu-8*, produced particularly low levels of the enzyme.

None of the mutants showed a deficiency in glyoxylate cycle enzymes, although *acu-10* (S10-3) demonstrated significantly more isocitrate lyase activity on sucrose than did the wild-type. During growth on acetate, the enzymes of the glyoxylate cycle are located in a specialized organelle, the glyoxysome, which is a representative of a subclass of microbodies found in *Neurospora* (Schwitzgubel *et al.*, 1981; Kionka & Kunau, 1985). We have also undertaken crude fractiona-

tion of these organelles (as necessary for the acetyl-CoA hydrolase assay) and found that all the mutants had intact glyoxysomes with their relative isocitrate lyase and malate synthase levels similar to wild-type. The mutants are apparently not deficient in organelle biogenesis or protein targeting.

The gluconeogenic enzymes fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, are induced non-coordinately with the glyoxylate cycle enzymes during growth on acetate (Beever, 1975). In all the mutant classes the two enzyme activities behaved as they do in the wild-type. The specific activity of NADP⁺-linked malic enzyme, which generates pyruvate from malate, declined in the mutants and the wild-type upon transfer to acetate. This was perhaps unexpected as in *Aspergillus nidulans* the enzyme is apparently derepressed and has been ascribed an anaplerotic function in the supply of pyruvate under conditions where pyruvate kinase activity is low (McCullough & Roberts, 1974; Kelly & Hynes, 1981). Unusually, a NADP⁺-specific malate dehydrogenase activity was also detected. This enzyme became derepressed on acetate in the majority of strains, the mutant *acu-11* (S30-12) being exceptional in that it showed a marked derepression on sucrose. The assay conditions for the malic enzyme and NADP⁺-dependent malate dehydrogenase were quite similar; therefore the reduction of NADP⁺ may represent a compound activity. However, malic enzyme activity was also followed as the formation of pyruvate in this study, and as these enzyme activities appeared to decline and derepress in an antiparallel fashion, the malate dehydrogenase fraction may be distinguished.

In addition to derepression of NADP⁺-specific malate dehydrogenase on sucrose mutant *acu-11* (S30-12) also showed a lower level of the NADP⁺-specific enzyme on acetate. However, mutant *acu-13* (S30-9) failed to induce NADP⁺-specific malate dehydrogenase activity with acetate. This mutant was investigated further by the separation and visualization of malate dehydrogenase activities on 10% native polyacrylamide gels. The wild-type and all other mutant classes produced three isoenzymic activities, only one of which (the slowest migrating band) was significantly expressed on sucrose. The middle and fastest migrating bands became plainly visible only after acetate (or ethanol) induction (Fig. 2). In contrast, *acu-13* (S30-9) was deficient in activity of the middle band despite some derepression of the faster migrating band. Other alleles of *acu-13*, the most represented *fac*⁺ *acu* class, were also either reduced or deficient in this middle band.

The wild-type and all classes of mutant had inducible levels of glutamate:oxalacetate transaminase and NADP⁺-specific isocitrate dehydrogenase; the NADP⁺-dependent enzyme remained constitutive.

Table 3. *Enzyme activities in the wild-type and representative mutants from each complementation group of acu mutants*

Activities were measured after 22 h growth on sucrose medium (non-induced) and after a subsequent 6 h incubation in acetate medium (induced). Values are the means of several assays $\pm$ the standard deviations in parentheses, the figures corresponding to the last decimal place of the means.

Strain	Enzyme activity [$\mu\text{mol product min}^{-1}$ (mg protein) $^{-1}$]												
	PEP					NAD $^{+}$ -specific			NADP $^{+}$ -specific			Glutamate:	
	Acetyl-CoA synthetase	Acetyl-CoA hydrolase	Isocitrate lyase	Malate synthase	Malate carboxylase	Fructose-1,6-bisphosphatase	isocitrate dehydrogenase	isocitrate dehydrogenase	malate dehydrogenase	malate dehydrogenase	malate dehydrogenase	malate dehydrogenase	oxalacetate transaminase uptake*
<i>STa</i> (wild-type)													
Non-induced	0.021(3)	<0.001	0.3(2)	<0.01	1.33(9)	0.28(4)	0.056(8)	0.08(4)	1.65(15)	0.38(4)	1.563(13)	22(2)	
Induced	0.064(6)	0.065(5)	8.0(18)	0.14(6)	3.35(15)	0.35(5)	0.052(6)	0.22(4)	16.14(31)	2.66(12)	4.130(15)	188(6)	
<i>acu-10</i> (S10-3)													
Non-induced	0.052(6)	0.005(2)	1.4(2)	0.02(2)	1.33(9)	0.19(3)	0.043(9)	0.12(6)	1.93(9)	0.26(4)	0.871(13)	19(3)	
Induced	0.104(22)	0.075(2)	5.0(15)	0.14(4)	3.33(33)	0.26(4)	0.047(8)	0.04(4)	16.05(15)	5.66(6)	1.275(15)	96(9)	
<i>acu-11</i> (S30-12)													
Non-induced	<0.001	0.006(4)	0.1(3)	<0.01	1.26(3)	0.22(4)	0.047(3)	0.09(3)	1.80(16)	3.81(9)	1.252(16)	17(3)	
Induced	0.042(6)	0.054(8)	2.5(10)	0.40(5)	2.01(21)	0.24(4)	0.041(7)	0.26(4)	4.60(20)	3.80(8)	3.350(25)	16(2)	
<i>acu-12</i> (S10-4)													
Non-induced	0.027(3)	<0.001	0.5(2)	<0.01	1.50(9)	0.17(3)	0.035(5)	0.11(3)	3.10(15)	<0.01	1.431(17)	13(5)	
Induced	0.068(8)	0.009(3)	13.0(18)	0.06(3)	4.60(21)	0.19(3)	0.042(4)	0.46(6)	22.14(12)	4.2(4)	3.697(19)	117(13)	
<i>acu-13</i> (S30-9)													
Non-induced	0.039(3)	0.005(2)	0.4(1)	0.04(2)	1.90(5)	0.24(6)	0.068(8)	0.08(2)	1.50(10)	1.34(6)	0.555(15)	24(2)	
Induced	0.065(5)	0.044(6)	7.00(10)	0.30(6)	3.62(17)	0.21(7)	0.082(6)	0.31(7)	1.70(9)	2.16(8)	0.680(20)	171(17)	

* $10^{-2} \times$ Corrected counts min^{-1} per 4×10^4 conidia for the 16 min assay period.

Fig. 2. Native (10%) PAGE of crude enzyme extract, activity stained for NAD⁺-specific malate dehydrogenase. Lanes 1–3 and 4–6 represent wild-type (ST4) and *acu-13* (S30-9) activities respectively. Lanes 1 and 4 show activities after primary growth on sucrose (16 h), lanes 2 and 5 after subsequent transfer to ethanol (6 h) and lanes 3 and 6 after transfer to acetate (6 h).

Uptake experiments using [¹⁴C]acetate demonstrated that *acu-11* (S30-12) was unable to accumulate acetate. The other classes of mutant showed near wild-type levels of [¹⁴C]acetate accumulation over the 16 min assay period. The lack of uptake by *acu-11* (S30-12) could indicate a permease deficiency; however, it may equally reflect the inability of the mutant to metabolize and fix the labelled acetate. When uptake was assayed with shorter intervals (30 s), the initial rate of uptake was similar to wild-type. The substrate, however, was not retained to be fixed by metabolism. The primary destination of the acetate is potentially aberrant.

Discussion

Selection for mutants on the basis of fluoroacetate resistance resulted in the generation of four novel *acu* mutants, which may help to complete our understanding of the function and regulation of proteins involved in acetate metabolism in *Neurospora*. However, our study failed in its original aim to generate a pleiotropic mutant similar to *facB* found in *Aspergillus nidulans* (Apirion, 1965; Armitt *et al.*, 1976; Hynes, 1977; Hynes & Kelly 1977; Katz & Hynes, 1989). In the yeast *Yarrowia lipolytica*, however, the recently isolated *trans*-dominant GPR-1 mutant deficient in glyoxylate pathway regulation was found not to be fluoroacetate resistant (Kujau *et al.*, 1991). A putative *facB*-type mutant generated in this laboratory by duplication induced mutation was also fluoroacetate sensitive (N. E. Owen & I. F. Connerton, unpublished data). The *fac* mutants of *N. crassa* selected

here were initially chosen due to their vigorous growth and good condition, which may be reliant on a functional glyoxylate cycle, since there is an increase in isocitrate lyase at this stage in the vegetative life cycle of *Neurospora* (Turian & Matikian, 1966). Therefore, there may have inadvertently been a selection against certain types of *acu* mutants. In *Aspergillus*, the major class of fluoroacetate resistant mutations are the *facA* mutations deficient in acetyl-CoA synthetase; this class was not selected in our study. Oddly, the equivalent acetyl-CoA synthetase deficient class (*acu-5*) of *Neurospora* was not fluoroacetate resistant. In the basidiomycete *Coprinus cinereus*, fluoroacetate selection yielded mutations at only one locus, *acu-1* (Casselton & Casselton, 1974; Baptista-Ferreira & Casselton, 1983), which results in a deficiency in acetyl-CoA synthetase. Revertants of these mutations were isolated which conferred a temperature-sensitive enzyme phenotype (Vousden & Casselton, 1983). It was assumed that *acu-1* represented the structural gene for acetyl-CoA synthetase. However, the gene has recently been cloned by hybridization to the *N. crassa* and *A. nidulans* genes (Connerton *et al.*, 1990) and found not to complement *acu-1*; it appears that *acu-1* may be a regulatory mutation equivalent to *facB* in *Aspergillus* (Maconochie *et al.*, 1992). The variation in the mutant types observed may reflect physiological differences in the enzymes and the regulation to which they are subject, which will ultimately determine the sites of action of fluoroacetate and its adducts.

The *acu-8* and *acu-12* mutations result in similar growth properties, do not complement each other in heterokaryons and are affected in acetyl-CoA hydrolase. The mutations are closely linked on LGIIR (<2%), which even so suggests they are independent genes. It should be noted, however, that *acu-8* is a RIP derived mutation and the influence, if any, of the severe changes associated with this are unknown for closely linked genetic crosses. It is formally possible that the genes encode separate subunits of the same enzyme complex that may undergo interference. In *Saccharomyces cerevisiae*, however, the enzyme is composed of a single polypeptide chain (Lee *et al.*, 1989).

Mutant *acu-11* (S30-12) is unable to grow on acetate but is capable of slow growth on ethanol. As there is no other known way to utilize ethanol other than via acetate it must be assumed the strain is metabolically competent. Initially, a defect in an acetate permease was indicated, which would explain the phenotype. However, as the glyoxylate cycle enzymes induced as normal and initial uptake was found to be as wild-type, the lesion is probably not a permease but concerns an internal transport problem that is apparently acid pH sensitive. The low levels of NAD⁺-specific malate dehydrogenase found in this mutant may also be due to a reduction/

absence of a secondary metabolite required for full induction. The acetate permease mutant *acp⁻ⁱ* (Rao & Debusk, 1977) is also likely to carry a lesion other than in the permease, since it fails to grow on ethanol and is also sensitive to fluoroacetate. Thomas & Baxter (1987) found that this mutant did not undergo significant flux via the glyoxylate cycle when fed with [¹³C]acetate and examined by nuclear magnetic resonance.

Lesions resulting in non-functional structural proteins essential for the integrity of the glyoxysome might yield an *Acu⁻* phenotype, although peroxisome-less mutants isolated in the yeasts *Saccharomyces cerevisiae* and *Hansenula polymorpha* do not exhibit a specific *Acu⁻* phenotype; rather, they are affected in fatty acid metabolism dependent upon β -oxidation (Erdmann *et al.*, 1989; Cregg *et al.*, 1990). All four *Neurospora* mutants tested, corresponding to each of the complementation groups, produced intact glyoxysomes, containing normal complements of the enzymes isocitrate lyase and malate synthase.

The mutant *acu-13* (S30-9) did not induce NAD⁺-specific malate dehydrogenase and also had low levels of NADP⁺-specific malate dehydrogenase. *Neurospora* has three isoenzymic activities for NAD⁺-specific malate dehydrogenase that can be observed on activity gels, the two fastest migrating of which are induced upon transfer to acetate. Analysis of all the separate alleles of *acu-13* demonstrated either the loss or a reduction in intensity of the middle band upon transfer to acetate. At this stage it is not certain whether *acu-13* represents the structural gene for an acetate-inducible malate dehydrogenase or is pleiotropic to its induction. K. D. Munkres and colleagues have isolated mutants falling into two complementation groups (*ma-1* and *ma-2*), deficient in malate utilization in the presence of the *suc* (*ace-6*) mutation (deficient in pyruvate carboxylase and requiring TCA cycle intermediates for growth) that are altered in NAD⁺-specific malate dehydrogenase (Munkres *et al.*, 1965; Munkres & Richards, 1965). These mutations are thought to reside on either LGIII or LGVII, whereas *acu-13* is located on LGII. The acetate-inducible malate dehydrogenase has been located to the glyoxysome by Kobr *et al.* (1969), and Munkres *et al.* (1970) suggested that these isoenzymes may have subunits in common with those of the mitochondrial enzyme, which are altered in *ma-1* and *ma-2*. Further information as to the nature of these isoenzymes may become available shortly as we have recently cloned at least one malate dehydrogenase structural gene from *N. crassa* (P. T. Chaure & I. F. Connerton, unpublished).

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References

- APIRION, D. (1965). The two way selection of mutants and revertants in respect of acetate utilization and resistance to fluoroacetate in *Aspergillus nidulans*. *Genetics Research* **6**, 317–329.
- ARMITT, S., McCULLOUGH, W. & ROBERTS, C. R. (1976). Analysis of acetate non-utilizing (*acu*) mutants in *Aspergillus nidulans*. *Journal of General Microbiology* **92**, 263–282.
- BAPTISTA-FERREIRA, J. L. C. & CASSELTON, L. A. (1983). An allele map of the *acu-1* gene of *Coprinus cinereus*. *Revista de Biologia* **12**, 187–196.
- BARTH, G. (1985). Genetic regulation of isocitrate lyase in the yeast *Yarrowia lipolytica*. *Current Genetics* **10**, 119–124.
- BEEVER, R. E. (1975). Regulation of 2-phosphoenolpyruvate kinase and isocitrate lyase in *Neurospora crassa*. *Journal of General Microbiology* **86**, 197–200.
- CAMBARERI, E. B., JENSEN, B. C., SCHABTACH, E. & SELKER, E. U. (1989). Repeat-induced GC→AT mutations in *Neurospora*. *Science* **244**, 1571–1575.
- CASSELTON, L. A. & CASSELTON, P. J. (1974). Functional aspects of fluoroacetate resistance in *Coprinus* with special reference to acetyl-CoA synthetase deficiency. *Molecular and General Genetics* **132**, 255–264.
- CIONI, M., PINZANTI, G. & VANNI, P. (1981). Comparative biochemistry of the glyoxylate cycle. *Comparative Biochemistry and Physiology* **70B**, 1–26.
- CONNERTON, I. F. (1990). Premiotic disruption of the *Neurospora crassa* malate synthase gene by native and divergent DNAs. *Molecular and General Genetics* **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*. *Molecular Microbiology* **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. *Journal of General Microbiology* **138**, 1797–1800.
- CREGG, J. M., VAN DER KLEI, I. J., SULTER, G. J., VEENHUIS, M. & HARDER, W. (1990). Peroxisome-deficient mutants of *Hansenula polymorpha*. *Yeast* **6**, 87–97.
- DIXON, G. H. & KORNBERG, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochemical Journal* **72**, 3p.
- ERDMANN, R. M., VEENHUIS, M., MERTENS, D. & KUNAU, W.-H. (1989). Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 2432–2436.
- FLAVELL, R. B. & FINCHAM, J. R. S. (1968a). Acetate non-utilizing mutants of *Neurospora crassa*. 1. Mutant isolation, complementation studies and linkage relationships. *Journal of Bacteriology* **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. *Journal of Bacteriology* **95**, 1063–1068.
- HYNES, M. J. (1977). Induction of the acetamidase of *Aspergillus nidulans* by acetate metabolism. *Journal of Bacteriology* **131**, 770–775.
- HYNES, M. J. & KELLY, J. M. (1977). Pleiotropic mutants of *Aspergillus nidulans* altered in carbon metabolism. *Molecular and General Genetics* **150**, 193–204.
- JONES, M. E. & LIPMAN, F. (1955). Aceto-CoA-kinase. *Methods in Enzymology* **1**, 585–591.
- KATZ, M. E. & HYNES, M. J. (1989). Isolation and analysis of the acetate regulatory gene *facB*, from *Aspergillus nidulans*. *Molecular and Cellular Biology* **12**, 5696–5701.
- KELLY, J. M. & HYNES, M. J. (1981). The regulation of phosphoenolpyruvate carboxykinase and NADP-linked malic enzyme in *Aspergillus nidulans*. *Journal of General Microbiology* **123**, 371–375.
- KIONKA, C. & KUNAU, W.-H. (1985). Inducible β -oxidation pathway in *Neurospora crassa*. *Journal of Bacteriology* **161**, 153–157.
- KOBR, M. J., VANDERHEAGH, F. & COMBEPINE, G. (1969). Particulate enzymes of the glyoxylate cycle in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **37**, 640–645.
- 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.

- KUN, E., KIRSTEN, E. & SHARMA, M. L. (1977). Enzymatic formation of glutathione-citryl thioester by a mitochondrial system and its inhibition by (-)erythrofluorocitrate. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 4942-4946.
- LEE, F.-J. S., LIN, L.-W. & SMITH, J. A. (1989). Purification and characterization of an acetyl-CoA hydrolase from *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **184**, 21-28.
- MACONOCHE, M. K., CONNERTON, I. F. & CASSELTON, L. A. (1992). The *acu-1* gene of *Coprinus cinereus* as a regulatory gene required for induction of utilisation of enzymes. *Molecular and General Genetics* **234**, 211-216.
- MCCULLOUGH, W. & ROBERTS, C. F. (1974). The role of malic enzyme in *Aspergillus nidulans*. *FEBS Letters* **41**, 238-242.
- 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. *Molecular and Cellular Biology* **10**, 2638-2644.
- MUNKRES, K. D. & RICHARDS, F. M. (1965). Genetic alterations of *Neurospora* malate dehydrogenase. *Archives of Biochemistry and Biophysics* **109**, 457-465.
- MUNKRES, K. D., GILES, N. H. & CASE, M. E. (1965). Genetic control of *Neurospora* malate dehydrogenase and aspartate aminotransferase. *Archives of Biochemistry and Biophysics* **109**, 397-403.
- MUNKRES, K. D., BENVENISTE, K., GORSKI, J. & ZUICHES, C. A. (1970). Genetically induced subcellular mislocation of *Neurospora* mitochondrial malate dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America* **67**, 263-270.
- PERKINS, D. D. (1964). Multiple interchange stocks for linkage detection. *Neurospora Newsletters* **6**, 22-23.
- PETERS, R. A. (1957). Mechanism of the toxicity of the active constituent of *Dichapetalum cymosum* and related compounds. *Advances in Enzymology* **18**, 113-159.
- RAO, T. K. & DEBUSK, A. G. (1977). An inducible acetate transport system in *Neurospora crassa* conidia. *Biochimica et Biophysica Acta* **470**, 475-483.
- SELKER, E. U., CAMBARERI, E. B., JENSEN, B. C. & HAACK, K. R. (1987). Rearrangement of duplicated DNA in specialized cells of *Neurospora*. *Cell* **51**, 741-752.
- SCHWITZGUEBEL, J. P., MOLLER, I. M. & PALMER, J. M. (1981). Changes in density of mitochondria and glyoxysomes from *Neurospora crassa*: a re-evaluation utilizing silica sol gradient centrifugation. *Journal of General Microbiology* **126**, 289-295.
- TATUM, E. L., BARRATT, R. W. & CUTTER, V. M. (1949). Chemical induction of colonial paramorphs in *Neurospora* and *Syncephelastrium*. *Science* **109**, 509-511.
- THOMAS, G. H. & BAXTER, R. L. (1987). Analysis of mutational lesions of acetate metabolism in *Neurospora crassa* by ¹³C-nuclear magnetic resonance. *Journal of Bacteriology* **169**, 359-366.
- 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*. *Molecular Microbiology* **2**, 599-606.
- TURIAN, G. & MATIKIAN, N. (1966). Conidiation of *Neurospora crassa*. *Nature, London* **212**, 1067-1078.
- VOGEL, H. J. (1956). A convenient growth medium for *Neurospora* (medium N). *Microbial Genetics Bulletin* **13**, 42-43.
- VOUSDEN, K. H. & CASSELTON, L. A. (1983). Suppression of acetate mutants in *Coprinus cinereus*. II. Correlation of recessiveness and dosage with suppressed enzyme level. *Current Genetics* **7**, 385-392.
- WESTERGAARD, M. & MITCHELL, H. K. (1947). A synthetic medium favouring sexual reproduction. *American Journal of Botany* **34**, 573-577.