Enzymes of the 3-Oxoadipate Pathway in Fungi: A Serological Study of 3-Carboxymuconate Cyclase (Lactonizing Enzyme) in Fungi and its Possible Taxonomic Significance

By K. A. COOK* AND R. B. CAIN

Biological Laboratories, University of Kent, Canterbury, Kent CT2 7NJ

(Received 15 April 1977)

The immunoglobulin fraction of antiserum prepared against a homogeneous preparation of 3-carboxymuconate cyclase from *Aspergillus niger* L6 was used to study the serological relatedness of the enzyme in crude extracts of other fungi. Immunodiffusion and immunoelectrophoresis experiments confirmed the homogeneity of the pure *A. niger* L6 cyclase, its serological identity with the same enzyme in other strains of *A. niger* and the complete absence of any cross-reaction with the analogous 3-carboxymuconate cycloisomerase from *Pseudomonas putida* and *Nocardia opaca*. Extensive cross-reactions were observed with the cyclases from other species of *Aspergillus* and *Penicillium*. When such cross-reactions were examined quantitatively by the precipitin technique, several genera believed to be taxonomically related to *Aspergillus* and *Penicillium* on morphological grounds showed cross-reacting cyclases. Within the genera there was a broad correlation of serological relationships with morphological subdivisions. These correlations were considerably better than can be achieved by determinations of DNA base compositions because most of the genera examined have GC ratios of 50 to 55 mol %. Crude extracts of two taxonomically unrelated Basidiomycetes showed no serological relatedness to the antisera.

INTRODUCTION

The 3-oxoadipate pathway is utilized by bacteria and fungi for the transformation of catechol, protocatechuate and their respective precursors to succinate and acetyl-CoA. The biochemistry of the pathway is similar in both groups of micro-organisms, although in all fungi the subsequent metabolism of the ring cleavage product, 3-carboxymuconate, proceeds through 3-carboxymuconolactone (Gross, Gafford & Tatum, 1956; Cain, Bilton & Darrah, 1968*a*) rather than through the 4-substituted isomer as in bacteria (Stanier & Ornston, 1973).

The enzymes effecting lactonization of 3-carboxymuconate, which are called cycloisomerases in bacteria and cyclases in fungi to distinguish their mode of lactonization (I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature, 1972), have been purified from *Pseudomonas putida* (Patel, Meagher & Ornston, 1973) and *Aspergillus niger* (Thatcher & Cain, 1974*a*); they differ substantially in their amino-acid composition and quaternary structure and in many kinetic properties. The majority of fungi produce 3-carboxymuconate cyclases with essentially similar kinetic properties though the enzyme in Basidiomycetes disaggregates rather easily from the normal multimeric form (R. B. Cain, A. Columbi & B. A. Halsall, unpublished work). To find out if this similarity in catalytic properties extended to the enzyme structure, we utilized an antiserum, prepared against a homogeneous

* Present address: Microbiology Division, Shell Biosciences Laboratory, Shell Research Limited, Sittingbourne, Kent ME9 8AG.

preparation of 3-carboxymuconate cyclase from *A. niger* L6 (Thatcher & Cain, 1974a), to examine the extent of antigenic homology of this enzyme in fungi capable of degrading aromatic compounds and to confirm the anticipated serological dissimilarity of the fungal and bacterial enzymes.

METHODS

Organisms. *Aspergillus niger* L6, *Penicillium puberulum* B3 and *Penicillium crustosum* P6 (CMI191672) were isolated from soil and *Schizophyllum commune* from rotting wood by Dr B. Halsall (Cain *et al.*, 1968a). The two penicillia were identified by Dr A. H. S. Onions (Commonwealth Mycological Institute, Kew, Surrey). Other strains of *A. niger* used were CMI45551, one designated 'Newcastle' (from Dr C. H. Dickinson, Department of Plant Biology, University of Newcastle upon Tyne) and one designated 'Birkbeck' (from Dr Jane North, Department of Biological Sciences, City of London Polytechnic, London E1 7NT). Strain PL30 was a temperature-sensitive mutant derived from *A. niger* L6 by treatment with β -propiolactone (E. A. Ahlquist, unpublished results). It produced a catalytically active 3-carboxymuconate cyclase when grown at 25 °C or below but not at 30 °C. *Rhodotorula mucilaginosa* and *Aureobasidium pullulans* were isolated as aerial contaminants on *p*-hydroxybenzoate medium (Cain *et al.*, 1968a) and identified by the Brewing Industry Foundation, Nutfield, Surrey. *Aspergillus oryzae* and *Aspergillus nidulans* var. *albus* were soil isolates provided by Dr C. H. Dickinson; *A. nidulans* strain 224 was provided by Professor A. T. Bull (University of Kent, Canterbury); *Penicillium chrysogenum* w154-1255 (Righelato *et al.*, 1968) and *Penicillium nigricans* CMI39767 were from Dr A. Trinci (Department of Microbiology, Queen Elizabeth College, University of London). All other fungi were obtained from the Commonwealth Mycological Institute.

Pseudomonas putida A3.12 (ATCC12633; NCIB9494) was obtained from the National Collection of Industrial Bacteria. *Nocardia opaca* CA4 was the strain used by Cain, Tranter & Darrah (1968b) and by Rann (1973).

Media. Fungi were grown on the medium of Cook (1974) supplemented with 10 mM-glucose and 10 mM-*p*-hydroxybenzoate. This medium supported excellent mycelial growth and gave good activities of the cyclase in extracts. Bacteria were grown on the medium of Ornston & Stanier (1966) with 10 mM-*p*-hydroxybenzoate as inducer.

Growth and maintenance of cultures. All mycelial fungi were maintained by monthly subculture on either *p*-hydroxybenzoate agar or Oxoid potato dextrose agar slopes at 30 °C unless otherwise stated. *Pseudomonas putida* and *N. opaca* were similarly maintained on either *p*-hydroxybenzoate agar or Oxoid nutrient agar slopes. Yeasts were maintained on slopes of MYGP medium of Cain *et al.* (1968a).

Liquid cultures were grown in 1 l of glucose plus *p*-hydroxybenzoate medium in 2 l Erlenmeyer flasks incubated at the appropriate temperature on a rotary shaker operated at 140 rev. min⁻¹. Inocula for these cultures were grown for 36 to 48 h in 50 ml of basal medium containing only 10 mM-glucose as carbon source; spore inocula were used where possible, but otherwise inocula were mycelial suspensions prepared by homogenizing mycelium cut with a sterile cork-borer from the edge of colonies growing on potato dextrose agar plates. Utilization of the aromatic substrate was monitored spectrophotometrically by measuring the decrease in absorbance at 245 nm due to *p*-hydroxybenzoate, using a Pye Unicam SP 500 spectrophotometer. Cultures were harvested when the aromatic substrate had decreased by 75 to 80 %. This took about 30 h for rapidly growing species such as *Gliocladium deliquescens* or *Rhodotorula mucilaginosa* but up to 4 days for *Penicillium commune*. At these times the aromatic substrate was being most rapidly consumed and enzyme activities were at a maximum. In shaken cultures, *Kernia nitida* CMI151087 grew very slowly and it was therefore grown in stationary culture in 100 ml of glucose liquid medium in 1 l Roux bottles laid flat to increase the surface area. After a firm mycelial mat had formed (6 to 7 days at 30 °C), the medium was carefully decanted, the mat was rinsed with sterile distilled water, and sterile phosphate buffer, pH 6.0, containing 10 mM-*p*-hydroxybenzoate was introduced into the bottle. The refloat mycelial mat began to utilize the aromatic substrate within 36 h and was harvested when 80 % of this substrate had been consumed.

In some experiments, mycelial fungi were grown with 10 mM-protocatechuate or 2 mM-catechol (sterilized by filtration) instead of *p*-hydroxybenzoate.

Preparation of cell-free extracts. Mycelial fungi were harvested by filtration through muslin and the resulting pads of mycelium were dried between paper towels and stored at -30 °C if not required immediately. Extracts were prepared by grinding the mycelial pads to a fine powder under liquid nitrogen in a mortar; occasionally acid-washed sand was used as an abrasive. The ground mycelium, after thawing to 5 °C, was extracted with 2 vols potassium phosphate buffer (0.05 M, pH 6.8), and the extract was rapidly frozen and thawed twice. Mycelial debris was removed by centrifuging at 38000 g for 15 min.

Pseudomonas putida, *N. opaca*, *R. mucilaginosa* and *Aur. pullulans* were harvested by centrifugation and disrupted ultrasonically in potassium phosphate buffer (0.05 M, pH 6.8) as described by Cook & Cain (1974). Extracts were usually tested immediately but otherwise were stored at -30 °C until required. There was a negligible loss of cyclase activity at this temperature over 6 months in extracts of most fungi. However,

extracts from *Gliocladium roseum* and *G. deliquescens* were much less stable and were always used immediately.

Enzyme assays. 3-Carboxymuconate cyclase [3-carboxymuconolactone lyase (decyclizing), EC 5.5.1.5], 3-carboxymuconate cycloisomerase [4-carboxymuconolactone lyase (decyclizing), EC 5.5.1.2] and *cis-cis*-muconate cycloisomerase [4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing), EC 5.5.1.1] were assayed according to Cook & Cain (1974) and Ornston & Stanier (1966). A unit of activity for these enzymes corresponds to the disappearance of 1 μ mol of 3-carboxymuconate or *cis-cis*-muconate min^{-1} .

Protein was determined by the biuret method of Gornall, Bardawill & David (1949).

Preparation of antiserum. The immunizing antigen was 3-carboxymuconate cyclase from *A. niger* L6 [270 units (mg protein) $^{-1}$]. The enzyme was purified to homogeneity as described by Thatcher & Cain (1974a) but with the addition of a final chromatographic treatment on a column (45 $\times$ 2.5 cm diam.) of Sepharose 4-B (Pharmacia) equilibrated in potassium phosphate buffer (0.025 M, pH 7.6). The purified enzyme was dialysed for 60 h against physiological saline [0.85 % (w/v) NaCl], diluted in saline to 500 μ g ml^{-1} and stored at -30°C as 0.2 ml portions. Each portion contained sufficient enzyme (100 μ g) for one injection.

Antiserum was prepared in 10-week-old female New Zealand white rabbits; each animal received four intramuscular injections at 3 day intervals followed, after 4 days, by two intravenous injections 3 days apart. The intramuscular injections consisted of enzyme (100 μ g) diluted to a convenient volume (0.4 ml) with saline and emulsified with an equal volume of Freund's complete adjuvant. Intravenous injections were performed similarly but without adjuvant. Six days after the second intravenous injection the animal was test bled for confirmation of immunological purity by preliminary immunodiffusion experiments, then given a final intravenous injection and bled 1 week later. The total amount of enzyme administered to each rabbit was thus 700 μ g (400 μ g intramuscularly and 300 μ g intravenously).

Antiserum against 3-carboxymuconate cycloisomerase from *Ps. putida* was prepared as described by Stanier *et al.* (1970) using as the immunizing antigen a partially purified enzyme sample [66 units (mg protein) $^{-1}$, purified to stage 6 of the method of Patel *et al.* (1973)] provided by Dr L. N. Ornston.

Antibody preparation. IgG immunoglobulin was isolated from sera by repeated precipitation with finely ground solid ammonium sulphate added to 40 % saturation at room temperature. The fourth precipitate was dissolved in borate-buffered saline, exhaustively dialysed against the same buffer and centrifuged (1400 g for 30 min). The final supernatant was adjusted to 16 mg protein ml^{-1} and stored at -30°C until required (Campbell *et al.*, 1970). Immunoglobulin isolated from antiserum prepared against *A. niger* L6 3-carboxymuconate cyclase and *Ps. putida* 3-carboxymuconate cycloisomerase will subsequently be referred to as anti-cyclase IgG and anti-cycloisomerase IgG respectively, and that isolated from normal serum as normal IgG.

Immunodiffusion. This was carried out on microscope slides (2.5 $\times$ 7.6 cm) using the Ouchterlony double-diffusion technique (Stollar & Levine, 1963). Each slide was evenly coated with 2 ml of agar solution comprising 1 % (w/v) Noble agar (Difco) and 0.1 % (w/v) sodium azide in Sorensen's buffer (0.05 M, pH 7.2), and the agar was allowed to harden for 30 min. A punch was used to cut a matrix of six wells (2 mm diam.) spaced at a distance of 3 mm around a centre well and 3 mm apart.

In all experiments the centre well contained 3 μ l of purified antibody protein (16 mg ml^{-1}) and each antigen well contained 3 μ l of pure enzyme or cell-free extract from a test organism. All crude extracts were concentrated by freeze-drying or ultrafiltration and adjusted to a standard activity of 50 units of enzyme per ml of extract. The loaded gels were held in a humidifier at 4°C for 36 h and then examined. Those required for permanent record were washed with several changes of saline to remove unreacted protein, dried at room temperature, stained with acid fuchsin (Campbell *et al.*, 1970) and photographed.

Immuno-electrophoresis. Immuno-electrophoresis was performed on microscope slides as described by Campbell *et al.* (1970) using a flat-bed electrophoresis apparatus (Shandon Southern Instruments, Camberley, Surrey) at 10 V cm^{-1} applied for 1 h. Immediately after electrophoresis a trough was cut in the agar (60 $\times$ 1 mm), filled with the appropriate IgG (50 μ l), and the gels were developed, dried, stained and photographed as in a normal immunodiffusion experiment. Occasionally larger glass slides (12 $\times$ 5.2 cm) were used to compare immuno-electrophoretic behaviour of four or more strains together. Such slides required three or more parallel troughs for antibody administration and 20 to 25 V cm^{-1} applied for 2 to 3 h.

Titration of enzyme with antibody. The affinity of *A. niger* L6 anti-cyclase IgG for various cycloisomerases or cyclases was quantitatively determined by titrating the enzyme with antibody within the range where the activity decreased linearly with increase in antibody amount. A crude cell-free extract (100 μ l) of the test organism was incubated with dilutions of anti-cyclase IgG in a final volume of 200 μ l. Dilutions of the specific antibody were made with normal IgG (16 mg protein ml^{-1}). Reaction mixtures were incubated at 37°C for 30 min in 500 μ l Beckman microfuge tubes and then further incubated at 4°C for 16 h. Any precipitated antigen-antibody complex was removed by centrifuging at 12000 g for 5 min (Beckman 152 microfuge) and the supernatant was assayed for residual 3-carboxymuconate cyclase or cycloisomerase activity.

The equivalence point, defined as munits of enzyme precipitated per μg of antibody added, was determined from the slope of a plot of residual enzyme activity in the supernatant (units ml^{-1}) against the amount of anti-cyclase IgG used (μg). The degree of cross-reactivity of the enzyme in an extract of a specific organism with the antibody prepared against *A. niger* L6 cyclase was calculated from its observed equivalence point expressed as a percentage of that obtained with the homologous enzyme from *A. niger* L6.

Modifications required for experiments with the Gliocladium enzyme. *Gliocladium roseum* grew slowly with poor mycelial yields and enzyme activity whereas *G. deliquescens* grew rapidly and gave extracts with high cyclase activity. Titration of the enzyme in extracts of either species with anti-cyclase IgG or even with normal IgG, prepared in borate-buffered saline, left no residual cyclase activity. This inactivating effect was traced to the saline content of the immunoglobulin preparations (see Results) to which the *Gliocladium* cyclase was particularly sensitive. Samples of anti-cyclase IgG and normal IgG from the ammonium sulphate precipitation steps were therefore dissolved in borate buffer (0.05 M, pH 8.5), rather than in borate-buffered saline, dialysed against the same buffer to remove ammonium sulphate and adjusted to a protein concentration of 16 mg ml^{-1} . Immunotitrations with these saline-free preparations always included positive controls of *A. niger* L6 extract, also prepared without saline, to permit strict comparison with the homologous cyclase.

RESULTS

Specificity of A. niger L6 anti-cyclase IgG and *Ps. putida* anti-cycloisomerase IgG

When an electrophoretically pure preparation of 3-carboxymuconate cyclase from *A. niger* L6, and a crude extract of the same fungus grown with *p*-hydroxybenzoate were tested in immunodiffusion experiments with *A. niger* L6 anti-cyclase IgG, single sharp precipitin lines were formed against both preparations (Fig. 1*a*); these lines coalesced without spurring indicating identity of the two antigens. A single precipitin line was similarly observed when crude extract from *A. niger* L6 grown with protocatechuate was used as antigen. The single precipitin lines suggested that the antibody was immunologically pure; this was confirmed by immunoelectrophoresis of the pure enzyme. No precipitin lines were evident against extracts of *A. niger* L6 grown with either catechol or glucose (Fig. 1*a*), nor were there any non-specific reactions evident when normal IgG was used as antiserum.

The anti-cyclase IgG showed no cross-reaction with purified 3-carboxymuconate cycloisomerase from *Ps. putida* nor with crude extracts of *Ps. putida* or *N. opaca* grown with *p*-hydroxybenzoate and concentrated to contain at least 50 units of the enzyme per ml of extract (Fig. 1*d*). Conversely, the anti-cycloisomerase IgG derived from *Ps. putida* gave no cross-reactions on immunodiffusion slides with pure *A. niger* L6 3-carboxymuconate cyclase or with crude extracts of *A. niger* grown on media which elicited high activities of the cyclase (Fig. 1*c*). There was likewise no cross-reaction between the anti-cycloisomerase IgG derived from *Ps. putida* and a crude extract of *N. opaca* grown with *p*-hydroxybenzoate or protocatechuate, confirming the heterologous nature of the cycloisomerases in these two bacteria.

Before using the *A. niger* L6 anti-cyclase IgG in experiments with other fungi, the identity of the immunological response was confirmed with the Newcastle and Birkbeck strains of *A. niger* where single sharp precipitin lines coalesced perfectly with that given by an extract of induced *A. niger* L6 and showed no spurring (Fig. 1*b*). Extracts from *A. niger* CMI45551 showed a more diffuse precipitin line but this also coalesced with that formed against the type strain with no evident spurring, indicating homology. No cross-reaction was shown with extracts of the mutant PL30 grown at 30 °C (Fig. 1*a*), suggesting the absence of a protein immunologically related to the active cyclase.

The immunological identity of the cyclases from *A. niger* L6 and the other strains of *A. niger* was confirmed by immunoelectrophoresis of all four *A. niger* strains run concurrently, both separately and as a mixture, on one large glass slide at pH values 7.7, 8.5 and 9.5. Under these conditions all four enzymes moved an identical distance towards the anode at each pH value during electrophoresis and the arcs of precipitation showed identity of reaction in each case.

Fig. 1. Detection of 3-carboxymuconate cyclase by cross-reaction with purified anti-cyclase IgG to *A. niger* L6. All immunodiffusion experiments were carried out on slides, washed with saline, stained with carbol fuchsin and photographed. Wells are numbered clockwise 1 to 6 beginning with the uppermost and the centre well contained the antiserum.

(a) Immunological specificity of the purified anti-cyclase IgG. Wells contained: 1, pure cyclase from *A. niger* L6; extracts of 2, *A. niger* L6 grown with glucose; 3, *A. niger* L6 grown with catechol; 4, *A. niger* L6 grown with protocatechuic acid; 5, *A. niger* mutant PL30 (temperature-sensitive cyclase) grown at 30 °C with *p*-hydroxybenzoate; and 6, *A. niger* L6, grown with *p*-hydroxybenzoate.

(b) Immunological identity of *A. niger* strains from various sources. Wells contained: 1, pure cyclase from *A. niger* L6; extracts of 2, *A. niger* CM145551; 3, *A. niger* Birkbeck strain; 4, *A. niger* L6; 5, *A. niger* Newcastle strain; and 6, *A. niger* L6. All crude extracts were derived from cultures grown with *p*-hydroxybenzoate. The centre well contained purified anti-cyclase IgG to *A. niger* L6.

(c) Immunological dissimilarity of 3-carboxymuconate cycloisomerase from *Ps. putida* and 3-carboxymuconate cyclase from *A. niger* L6 measured against the anti-cycloisomerase IgG to *Ps. putida* in the centre well. Wells contained: 1, partially purified cycloisomerase from *Ps. putida*; 2 and 5, pure cyclase from *A. niger* L6; 3 and 6, extracts of *A. niger* L6 grown with *p*-hydroxybenzoate; and 4, extract of *Ps. putida* grown with *p*-hydroxybenzoate.

(d) Immunological dissimilarity of 3-carboxymuconate cyclases from *A. niger* L6 and the other fungi and 3-carboxymuconate cycloisomerases from *Ps. putida* and *N. opaca*, measured against the anti-cyclase IgG to *A. niger* L6 in the centre well. All cultures were grown with *p*-hydroxybenzoate. Wells contained: 1, pure cyclase from *A. niger* L6; extracts of 2, *Paecilomyces variotii*; 3, *Penicillium terrestre*; 4, *A. niger* L6; 5, *Ps. putida*; and 6, *N. opaca*.

(e) Immunological cross-reactions between the pure anti-cyclase IgG to *A. niger* L6 (in the centre well) and the cyclase in other species of *Aspergillus* grown with *p*-hydroxybenzoate. Wells contained: 1, pure cyclase from *A. niger* L6; extracts of 2, *A. rugulosus*; 3, *A. nidulans* var. *albus*; 4, *A. niger* L6; 5, *A. terreus*; and 6, *A. fischeri* var. *glaber*.

(f) Immunological cross-reactions between the pure anti-cyclase IgG to *A. niger* L6 (in the centre well) and the cyclase in species of *Aspergillus* and other genera grown with *p*-hydroxybenzoate. Wells contained: 1, pure cyclase from *A. niger* L6; extracts of 2, *A. amstelodami*; 3, *Trichoderma viride*; 4, *A. niger* L6; 5, *P. crustosum* F6; and 6, *P. puberulum* B3.

Immunodiffusion experiments with other species of Aspergillus

A range of aspergilli (including species known to have ascosporic as well as conidial stages) which utilized *p*-hydroxybenzoate and formed 3-carboxymuconate cyclase (Table 1) were examined by immunodiffusion against anti-cyclase IgG. In all experiments the centre well contained *A. niger* L6 anti-cyclase IgG and the upper and lower wells contained pure *A. niger* L6 cyclase and *A. niger* crude extract respectively. This enabled direct comparison of cross-reactions between *A. niger* and four other *Aspergillus* species to be made on one slide. All the *Aspergillus* species examined gave extracts which showed marked heterology with the *A. niger* L6 enzyme; single precipitin lines were formed with heavy spurs directed towards the heterologous antigen well and the precipitin lines frequently did not coalesce with those formed against *A. niger* cyclase (Fig. 1*e, f*). With three species (*A. amstelodami*, *A. fischeri* var. *glaber* and *A. fumigatus*) from morphological section I (Raper & Fennell, 1965), no cross-reaction was observed.

Immunodiffusion experiments with species of Penicillium and other fungi

Halsall (1971) established that the 3-carboxymuconate cyclases present in several penicillia had similar molecular weights and kinetic properties to that found in *A. niger* so we extended our immunological examination of the fungal cyclases to include those from selected species of the genus *Penicillium* and other related genera (Table 3). Species such as *P. capsulatum*, *P. chrysogenum*, *P. crustosum* P6, *P. nigricans*, *P. puberulum* B3 and *P. rugulosum* gave clear cross-reactions by immunodiffusion although marked heterology was evident; there was heavy spurring, diffuse arcs and no coalescence of the precipitin lines with *A. niger* L6 control extract. (Fig. 1*f*). Extracts of *P. commune*, *P. frequentans* and *P. terrestre* gave just perceptible but very diffuse precipitin reactions only when concentrated to more than 50 units of cyclase ml⁻¹ (Fig. 1*d*), while *P. albicans* showed no cross-reaction.

No observable immunodiffusion cross-reactions were obtained using the anti-cyclase IgG with any other fungal extract, even though several of the genera examined have, like *Aspergillus* and *Penicillium*, been placed in the Ascomycete families Aspergillaceae (Bessey, 1950), and Trichocomataceae (Malloch & Cain, 1972) or in the section Phialosporae of the Hyphomycetes (Barron, 1968).

Quantitative titration of the fungal 3-carboxymuconate cyclases with antibody

The antibody did not appear to bind at or near the catalytic site of the enzyme since no inhibition of total enzyme activity was observed when antibody and enzyme were incubated for up to 12 h before assaying. The enzyme-antibody complex so formed, however, could be removed from unreacted enzyme by centrifugation. Representative titration curves obtained with extracts from a number of *Aspergillus* species are shown in Fig. 2.

Determination of their equivalence points (Table 2) confirmed the close identity of the L6, Newcastle and Birkbeck strains indicated previously by immunodiffusion and immunoelectrophoresis. Strain CMI4555I, which had an element of dissimilarity in these tests, still showed over 80 % cross-reaction with L6, a value well above that found with any other *Aspergillus* species.

Among the other aspergilli, whether an ascosporic stage is known or not, most species produced a cyclase having 33 to 61 % cross-reaction with the anti-cyclase IgG; all of these fell into the broad morphological sections II and III of Raper & Fennell (1965). Of the remaining four species, all from section I, *A. penicilloides* (a strict osmophile) was unable to metabolize *p*-hydroxybenzoate but *A. amstelodami*, *A. fumigatus* and *A. fischeri* var. *glaber* formed an immunologically distinct group with less than 20 % cross-reaction; this degree of dissimilarity may have accounted for the failure of these three species to react in immunodiffusion experiments.

Table 1. Typical activities of 3-carboxymuconate cyclase in extracts of different fungi

The specific activities are those found in extracts of fungi harvested when 80 % of the aromatic substrate (*p*-hydroxybenzoate or protocatechuate) had been consumed. Datum for *K. nitida* was obtained with extracts of mycelium grown by the replacement technique (see Methods).

Organism	Specific activity [units (mg protein) ⁻¹]
<i>Aspergillus niger</i> L6	1.22
<i>A. niger</i> (Birkbeck)	1.30
<i>A. niger</i> (Newcastle)	1.03
<i>A. niger</i> CMI45551	0.95
<i>A. amstelodami</i> CMI17455	1.45
<i>A. fischeri</i> var. <i>glaber</i> CMI61447	1.51
<i>A. flavus</i> CMI910196	4.13
<i>A. nidulans</i>	1.96
<i>A. oryzae</i>	3.00
<i>A. penicilloides</i> CMI140816	0
<i>A. terreus</i> CMI126254	1.43
<i>A. sydowii</i> CMI63904	5.22
<i>Penicillium capsulatum</i> CMI40576	0.52
<i>P. commune</i> CMI39812ii	0.28
<i>P. terrestre</i> CMI111867	0.17
<i>Gliocladium roseum</i> CMI40024ii	0.11
<i>G. deliquescens</i> CMI101525	1.28
<i>Cladosporium resinae</i> CMI88968	2.71
<i>Kernia (Magnusia) nitida</i> CMI151087	0.30
<i>Monascus ruber</i> CMI62570	0.66
<i>Paecilomyces variotii</i> CMI108007	1.33
<i>Penicillium clavariaeformis</i> CMI160389	1.23

Fig. 2. Quantitative immunotitration of 3-carboxymuconate cyclase in crude extracts of several aspergilli with anti-cyclase IgG. Titrations were carried out as described in Methods using 100 μ l portions of the appropriate extract and anti-cyclase IgG diluted with normal IgG where necessary. The extracts used were: ○, *A. flavus*; △, *A. amstelodami*; ■, *A. oryzae*; ●, *A. versicolor*; ▲, *A. niger* L6; and □, *A. niger* Birkbeck.

Table 2. *Cross-reactions of A. niger L6 anti-cyclase IgG with 3-carboxymuconate cyclase from Aspergillus species*

Equivalence points (munits of 3-carboxymuconate cyclase precipitated per μg of *A. niger* L6 anti-cyclase IgG) and the degree of cross-reaction occurring with the enzyme from heterologous sources were determined as described in Methods. The base compositions of fungal DNA are from Storck & Alexopoulos (1970). Morphology sections are based on the presence of uniseriate and biseriate sterigmata (Raper & Fennell, 1965; pp. 132–135).

Organism	mol % GC in DNA	Morphology section	Equivalence point	Cross- reaction (%)
<i>Aspergillus niger</i> L6	52.5–54	II	19.0	100
<i>A. niger</i> (Birkbeck)	52.5–54	II	22.0	115.8
<i>A. niger</i> (Newcastle)	52.5–54	II	17.2	90.5
<i>A. niger</i> CMI45551	52.5–54	II	15.4	81.3
<i>A. amstelodami</i> CMI17455	52–56	I	3.3	17.4
<i>A. fischeri</i> var. <i>glaber</i> CMI61447	51.5	I	2.5	13.1
<i>A. fumigatus</i> CMI143864	53	I	0.63	3.3
<i>A. candidus</i> CMI127260	53.5	II	9.0	47.3
<i>A. flavus</i> CMI910196	51	II	11.6	61.0
<i>A. oryzae</i>	52.5	II	6.3	33.1
<i>A. nidulans</i>	51–54	III	6.9	36.4
<i>A. nidulans</i> var. <i>albus</i>	—	III	7.7	40.5
<i>A. rugulosus</i> CMI153724	54	III	6.4	33.1
<i>A. sydowii</i> CMI63904	52.5	III	10.8	56.8
<i>A. terreus</i>	55	III	9.7	51.0
<i>A. versicolor</i> CMI91859	53.5	III	8.0	42.0

Most of the penicillia examined (Table 3) were species of the Asymmetrica and Biverticillata sections of the genus, which, with the exception of *P. commune*, formed an immunologically compact group whose cyclases all showed 37 to 47 % cross-reaction with the anti-cyclase IgG. Two species of the morphologically distinct Monoverticillata subgroup, *P. capsulatum* and *P. frequentans*, were also immunologically distinct with 27 % and 28 % cross-reacting cyclases, respectively, while *P. albicans*, a member of the Polyverticillata subgroup with many Scopulariopsis-like features (Raper & Thom, 1949) produced, like *Scopulariopsis candida*, a cyclase which showed less than 4 % cross-reaction.

Among other genera examined, those acknowledged on morphological and cultural criteria as having some relationship with *Aspergillus* and *Penicillium* all gave cyclases with a detectable cross-reaction with the anti-cyclase IgG (Table 3). In contrast, extracts of the Basidiomycetes *Schizophyllum commune* and *Rhodotorula mucilaginosa* (Banno, 1967), of *Trichoderma viride* and *Aureobasidium pullulans* and of the bacteria *Ps. putida* and *N. opaca*, showed no cross-reaction with the anti-cyclase IgG in these experiments.

Saline-sensitive 3-carboxymuconate cyclase from Gliocladium

Immunotitration of extracts of *G. deliquescens* and *G. roseum* with *A. niger* L6 anti-cyclase IgG led to complete loss of enzyme activity in the supernatants; a similar effect was observed when normal IgG was used, even when greatly diluted in borate-buffered saline (containing 145 mM-NaCl). As polyvalent anions inhibit some fungal cyclases (Thatcher & Cain, 1975) we suspected that borate was inhibiting the cyclase but eventually found that, unlike the cyclase of *A. niger*, the enzymes from *G. deliquescens* and *G. roseum* were sensitive to brief exposure to NaCl or KCl, 1.45 mM and 0.145 mM-NaCl causing 90.7 % and 37.6 % inhibition, respectively, of the cyclase. When preparations of both anti-cyclase IgG and normal IgG were made in borate buffer (see Methods) rather than in borate-buffered saline, normal

Table 3. Cross-reactions of *A. niger* L6 anti-cyclase IgG with 3-carboxymuconate cyclase from species of *Penicillium* and other genera

Conditions and calculations are as described in the legend to Table 2. The morphology sections used for the taxonomic classification of the penicillia are those of Raper & Thom (1949). The equivalence point of *A. niger* L6, titrated with anti-cyclase IgG prepared in borate-buffered saline, was 19.0.

Organism	Morphology section and subsection	mol % GC in DNA	Equivalence point	Cross-reaction (%)
A. <i>Penicillium frequentans</i>				
CM128043	Monoverticillata	51	5.3	28
<i>P. capsulatum</i> CM140576	Monoverticillata	51.5-52	5.2	27
<i>P. nigricans</i> CM139767	Asymmetrica-Divaricata	—	8.0	42
<i>P. commune</i> CM139812ii	Asymmetrica-Lanata	—	2.7	14.2
<i>P. chrysogenum</i>	Asymmetrica-Velutina	51-54	8.3	43.6
<i>P. crustosum</i> P6, CM1191672	Asymmetrica-Fasciculata	—	7.6	40
<i>P. puberulum</i> B3	Asymmetrica-Fasciculata	—	8.9	47
<i>P. terrestre</i> CM1111867	Asymmetrica-Funiculosa	—	7.1	37.3
<i>P. rugulosum</i> CM1134323	Biverticillata-Symmetrica	50.5-55	9.1	47.8
<i>P. albicans</i> CM163215	Polyverticillata	—	0.66	3.5
B. <i>Monascus ruber</i>				
CM162570		—	2.63	13.8
<i>Paecilomyces variotii</i> CM1108007		51.5	1.75	9.2
<i>Penicillium clavariaeformis</i> CM1160389		—	1.38	7.3
<i>Kernia (Magnusia) nitida</i> CM1151087		—	1.38	7.3
<i>Scopulariopsis candida</i> CM1129578		50	0.75	3.9
<i>Cladosporium resinae</i> CM188968		54	0.83	4.4
<i>Trichoderma viride</i>		49.5	0	0
<i>Aureobasidium pullulans</i>		51.5	0	0
C. <i>Rhodotorula mucilaginosa</i>				
<i>Schizophyllum commune</i>		61-63	0	0
		57-61	0	0
D. <i>Nocardia opaca</i> CA4				
<i>Pseudomonas putida</i> A3.12		62	0	0
		62.5	0	0

titration curves were obtained with both the *Gliocladium* species and with *A. niger* L6. The equivalence point for *A. niger* L6 extracts titrated with borate-buffered anti-cyclase IgG was 17.4, insignificantly different from values obtained previously in borate-buffered saline (Table 2). We therefore regard the results obtained for *G. deliquescens* (equivalence point 1.08; 6.2 % cross-reaction) and *G. roseum* (equivalence point 0.23, 1.3 % cross-reaction) as valid comparisons.

DISCUSSION

Fungal taxonomists have traditionally relied upon morphological characters, particularly the structure of sexual fruiting bodies, to establish their classifications. Indeed some have suggested that 'only morphological characters should be used to delimit species and all higher categories' (Snyder & Tousson, 1965), despite the fact that many of these features are based on subjective descriptions. This is particularly true of most of the genera examined in this paper, namely the form-genera within the Hyphomycetes (Barron, 1968; Kendrick & Carmichael, 1971) and the Plectomycetes which Fennell (1973) admitted was an 'unnatural class'.

An extensive analysis of the DNA base composition of fungi by Storck & Alexopoulos

(1970) showed that the whole subdivision Ascomycotina was encompassed within the range 48.5 to 60.0 mol % GC. Indeed, most species of the genera *Aspergillus* (*sensu* Raper & Fennell, 1965) and *Penicillium* (*sensu* Raper & Thom, 1949) (which include those ascogenic species now classified as *Eurotium*, *Emericella*, *Sartorya*, *Chaetosartorya*, *Talaromyces*, *Eupenicillium* and *Byssoschlamys*) have mol % GC contents of 53.5 ± 0.4 S.E.M. (range 48 to 61) and 51.5 ± 0.24 S.E.M. (range 47 to 60) respectively, so clearly cannot be distinguished by this method. For genetically based taxonomic relationships, DNA base pairing will probably ultimately provide the best approach (De Ley, 1968), but this has been little exploited by mycologists (Dutta & Ojha, 1972).

In the absence of distinct taxa based on nucleic acid composition, Wilson & Kaplan (1964) and Valen (1973) suggested that relationships between organisms could be revealed by comparing the structure of their enzymes since the amino-acid sequence of each enzyme would be a direct translation of the nucleotide sequence in a segment of the genetic material. Of the numerous protein analysis methods available, mycologists have made comparatively little use of serology as a taxonomic aid. Nowhere, for example, in monographs on the taxonomy of *Aspergillus* (Raper & Fennell, 1965) and *Penicillium* (Raper & Thom, 1949), is reference made to a single serological investigation, yet Mez (1929), with extraordinary foresight, was using serology to establish likely relationships between the Phycomycetes and Ascomycetes 25 years before the link between DNA and proteins was finally resolved. Even where the method has been tried, crude preparations of intact or ruptured spores and mycelia have been used as the antigen (Madhosingh, 1964; Amos & Burrell, 1967; Morton & Dukes, 1967; Biguet, Tran van Ky & Andrieu, 1966; Andrieu *et al.*, 1969), though the necessity for antibody, raised against a pure protein, was recognized by Imamoto, Yamane & Sueoka (1965) for quantitative comparison of homologous enzymes in crude extracts of related species.

The structural features of the proteins which determine why the bacterial cycloisomerase and fungal cyclase act on the same substrate yet produce isomeric products are still far from clear but serological investigations on mutant forms of the enzyme and on wild-type enzymes from related genera provide one insight into the problem. *Aspergillus niger* L6 was chosen for study because of its ubiquity (Raper & Fennell, 1965), its ability to grow rapidly upon several aromatic compounds (Cain *et al.*, 1968*a*), the availability of the homogeneous cyclase purified from it (Thatcher & Cain, 1974*a*) and the existence of several mutants with modified abilities to catabolize aromatic compounds (E. F. Ahlquist, unpublished results). We have confirmed the immunological dissimilarity of the *A. niger* L6 cyclase and the cycloisomerase from *Ps. putida* originally suspected from considerable differences in their amino-acid composition and quaternary structure (Patel *et al.*, 1973; Thatcher & Cain, 1974*b*). The 3-carboxymuconate cyclase and the *cis-cis*-muconate cycloisomerase of *A. niger* L6 (the latter is present in extracts of the fungus grown with 2 mM-catechol) were also serologically distinct – a feature noted in *Ps. putida* and *Acinetobacter calcoaceticus* (Patel *et al.*, 1973; Stanier *et al.*, 1970).

Cross-reactions within Aspergillus

Within the genus *Aspergillus* (including ascosporic species), four strains of *A. niger* were immunologically identical although one, the Birkbeck strain, was culturally quite distinct in producing yellow mycelium and secreting a yellow pigment in liquid media. The species with the closest immunological relationship to *A. niger* L6 was *A. flavus* (Table 2); these two species are regularly isolated from soil, both produce sclerotia, share the ability to attack many naturally-occurring aromatic compounds (Westlake & Simpson, 1961; Simpson, Narasimhachari & Westlake, 1963) and fall into section II of the classification of Raper & Fennell (1965), having biseriate or occasionally uniseriate sterigmata. *Aspergillus candidus*, also in section II, differs little from *A. niger* except for the absence of conidial pigmentation and roughening and showed considerable homology in its cyclase.

Many of the most widely distributed and well recognized species, all with strictly biserial sterigmata, belong to section III (Raper & Fennell, 1965). All had cyclases which fell within the range 33 to 57 % cross-reaction with the anti-cyclase IgG but since this range also spans values obtained with some section II species, our results suggest that aspergilli which at any time show biserial sterigmata should be classified together and that sections II and III could be merged. In contrast, section I species, all of which have strictly uniserial sterigmata, showed less than 20 % cross-reacting cyclases. They are therefore readily distinguishable morphologically and serologically from the other aspergilli. Species of this section also show osmophilic and thermotolerant properties which enable them to colonize substrates with low moisture content. *Aspergillus penicilloides* required such high sucrose concentrations for growth in liquid media that we were unable to induce aromatic degradation (Table 1).

Cross-reaction with other genera

Among bacterial cycloisomerases that have been examined serologically, interspecies dissimilarity was extensive (Stanier, 1967; Stanier *et al.*, 1970) and intergeneric cross-reaction absent (Patel *et al.*, 1973; Patel & Ornston, 1976). However, both immunodiffusion and quantitative precipitin methods revealed that most species of the Asymmetrica and Biverticillata-Symmetrica sections of *Penicillium* gave cyclases with 40 to 50 % cross-reaction (Table 3) and the two Monoverticillata species, *P. capsulatum* and *P. frequentans* produced cyclases with 27 to 28 % cross-reaction and so comprise serologically distinct groups. Raper & Thom (1949) recognized the uncertain taxonomic position of *P. albicans* when they placed it in their Polyverticillata section and drew attention to its *Scopulariopsis*-like character. This aberrant relationship was confirmed serologically by the similar weak cross-reacting pattern of the cyclases from *P. albicans* and *Scopulariopsis candida* (Table 3).

In addition to *Aspergillus* and *Penicillium*, Bessey (1950) included in his family Aspergillaceae the perithecial-forming genera *Monascus*, *Lilliputia* (conidial form *Gliocladium*), *Penicilliopsis* and *Magnusia* (*Kernia*). Raper & Thom (1949) believed *Gliocladium* to be closely related to *Penicillium* with *Paecilomyces* and *Scopulariopsis* further removed genetically. The close relationship of *Paecilomyces* and *Penicillium* was emphasized by Brown & Smith (1957) but *Paecilomyces*-like and *Penicillium*-like conidial stages have both been described for the thermotolerant, ascosporic genus *Talaromyces* (Stolk & Samson, 1971). Malloch & Cain (1972) pointed out that the name Aspergillaceae should apply only to Fungi Imperfecti and validated the family Trichocomataceae to include those Ascomycetes with *Aspergillus*, *Penicillium*, *Paecilomyces* and *Gliocladium* conidial states. Fennell (1973) nevertheless regarded this family as an artificial grouping and Barron (1968) placed these four form-genera and *Trichoderma* in his Phialosporae division of the soil-borne Hyphomycetes. Whether or not ascocarp or conidial morphology is regarded as the more important taxonomic feature, our results confirm that these genera (which incidentally have similar DNA base ratios) are, some albeit distantly, serologically related to *Aspergillus*. In contrast, the basidiomycete fungi *Schizophyllum commune* and *Rhodotorula mucilaginosa* have a much higher GC content (around 60 mol %) and their cyclases show no serological relationship to those of *A. niger*; the cyclases also differ substantially in their quaternary structure (R. B. Cain, A. Columbi & B. A. Halsall, unpublished results).

Therefore, within the Aspergillaceae (*sensu* Bessey, 1950), the results of our serological study confirm generic relationships and in *Aspergillus* and *Penicillium* broadly conform with intrageneric subdivisions based on morphological characters. The future use of the more sensitive micro-complement fixation technique (Kaplan, 1965) with many more species may better delimit intrageneric subdivisions and even species. We believe the use of 3-carboxymuconate cyclase as the antigen is justified because it is a uniquely fungal enzyme, unknown in prokaryotes or other eukaryotes, and has been found in over 40

genera of Phycomycetes, Ascomycetes, Basidiomycetes and Fungi Imperfecti (Cain *et al.*, 1968*a*; Halsall, Darrah & Cain, 1969). In the majority of these genera, the enzyme is stable for months in frozen extracts and raises good antibody titres.

The homology of the cyclase in fungal genera which, though similar in DNA base ratios, are distinguishable morphologically, implies considerable conservation of its protein structure. Such conservation is further supported by similar molecular weights, sub-unit sizes, kinetic constants, activation energies and pH optima of the cyclases from different genera (Halsall, 1971) and is perhaps not unexpected in an enzyme with strict specificity (Thatcher & Cain, 1975) for a substrate which can be derived from relatively few precursors. Fungi regulate the protocatechuate branch of the 3-oxoadipate pathway, which includes the cyclase, by substrate induction with either *p*-hydroxybenzoate or protocatechuate; the cyclase is always co-ordinately induced with 3-carboxymuconolactone hydrolase which removes the unique fungal metabolic product of cyclase action (Halsall, Darrah & Cain, 1969; Halsall, 1971; Cook & Cain, 1974; Huber *et al.*, 1975; E. F. Ahlquist and R. B. Cain, unpublished results). In soil such inducers probably derive from lignin (Henderson, 1961), flavonoids or alicyclic compounds like shikimate and quinate occurring in the litter from decomposing plant remains. Since many of the fungi examined in this paper occur in soil and plant litter (see Griffin, 1972; Dickinson & Pugh, 1974), the aromatic catabolic pathway which they have in common may itself have provided a selective force favouring evolution of the structure of a key catalytic protein and its conservation in genera that are quite distinct morphologically.

This study was supported by a grant from the Science Research Council (B/RG/3509) to R. B. Cain which is gratefully acknowledged.

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