Isolation and Haploidization of Heterozygous Diploid Strains in a Species of *Humicola*

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SUMMARY

Stable auxotrophic mutants were induced in *Humicola* sp. strain 20-31 by irradiation with far ultraviolet light. Mixed inoculation of different auxotrophs on to minimal medium agar produced prototrophic mycelia in all seven combinations tested with a frequency of 0.5 to 2.5 %. These prototrophic mycelia were stable when subcultured by macerated hyphae, single hyphal tips, single aleuriospores or single phialospores, and contained nuclei of approximately twice the volume of those in strain 20-31. They were more sensitive to growth inhibition by *p*-fluorophenylalanine (FPA) than 20-31 and its auxotrophic derivatives and, at concentrations of 140 and 160 µg FPA/ml, gave rise to more vigorously growing sectors. The mycelia from these sectors segregated for morphological and auxotrophic markers differentiating the parent auxotrophs and contained nuclei similar in size to strain 20-31. When reinoculated on to FPA media, these segregants showed the higher tolerance characteristic of strain 20-31 and failed to segregate further. It is concluded that in mixed culture, auxotrophic mutant derivatives of *Humicola* sp. strain 20-31 readily form somatic heterozygous diploids and that these are induced to haploidize by treatment with FPA. A difference in pigmentation which segregates spontaneously in strain 20-31 is attributed to a single chromosomal gene and the six markers studied are assigned to at least four linkage groups. Parasexual genetic analysis may be of value in taxonomic studies of imperfect fungi.

INTRODUCTION

A number of strains of the genus *Humicola* Traaen isolated from soil were observed to be unstable, giving rise to morphological variants either as sectors or single spore isolations (de Bertoldi, Lepidi & Nuti, 1972, 1973). An understanding of the nature and extent of this spontaneous variability was essential to the taxonomic review of the genus being conducted (de Bertoldi *et al.* 1972, 1973; Lepidi *et al.* 1972) and studies were therefore initiated to determine its genetic basis. This required the development of a system of genetic analysis in *Humicola* which is an Hypomycete. Genetic analysis is possible in filamentous fungi in the absence of sexual reproduction through the parasexual cycle (Pontecorvo, 1956; Pontecorvo & Käfer, 1958; Roper, 1966) and this method has been used in several imperfect fungi, including *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1954), *Aspergillus niger* (Lhoas, 1967) and *Verticillium albo-atrum* (Hastie, 1964). We report the isolation of heterozygous diploids from mixed cultures of marked derivatives of a strain of a *Humicola* sp. and the subsequent haploidization of these diploids. During haploidization, segregation and reassociation of the markers was observed.

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METHODS

Strains. All strains used were derived from strain 20-31 which was isolated from soil obtained from Sardinia (de Bertoldi & Verona, 1970). The morphological and cytological characteristics of this strain have been previously described (de Bertoldi *et al.* 1972; de Bertoldi, 1972). On the basis of mycelial and spore characters, strain 20-31 was referred to the genus *Humicola* but could not be assigned to any of the described species (de Bertoldi *et al.* 1972). It is included in a revision of the genus currently in progress (M. de Bertoldi and G. L. Hennebert, unpublished), when it will be formally described and identified. The original mass isolate of 20-31 produces abundant pigmented aleuriospores making the colonies appear black. Occasionally, cultures spontaneously produce white sectors which owe their distinctive appearance to a reduced level of aleuriospore production. Likewise, if 20-31 is propagated by single aleuriospores or phialospores the resulting colonies fall into black and white types, designated 20-31B and 20-31W respectively.

Cultural conditions. Minimal medium (MM) was basically that used for *Aspergillus nidulans* (Pontecorvo *et al.* 1953) except that 2 % (w/v) sucrose replaced 1 % (w/v) glucose as carbon source. Complete medium (CM) was prepared by the addition to 1 l MM of 1.0 g Difco Bacto Casamino acids, 1.0 g Difco Bacto peptone, 1.0 g Difco Bacto yeast extract, 0.15 g adenine and 20 ml of vitamin solution (Clowes & Hayes, 1968). MM and CM were solidified with 1.5 % (w/v) agar (Oxoid No. 3) to give MMA and CMA respectively. All incubation was at 26 °C and, except where stated, suspension and dilution of spores and mycelial fragments was carried out in 0.001 % (v/v) Tween 80 solution.

Production of spores. Strain 20-31 produces both aleuriospores and phialospores. The former are multinucleate with an average of ten nuclei per spore while the latter are uninucleate (de Bertoldi, 1972). For aleuriospore production, hyphal fragments were inoculated into 100 ml of liquid 2 % (w/v) malt + 2 % (w/v) yeast extract medium in 250 ml Erlenmeyer flasks and incubated for 7 days on a rotary shaker (2 inch throw, 220 rev./min). Cultures were filtered through glass wool to remove mycelial fragments and the aleuriospores collected and washed by centrifugation. Phialospores could only be obtained from MMA slide cultures produced by the method of Riddell (1950). After 7 days' incubation the mycelium on the cover slip was gently washed to remove the phialospores and the resulting suspension filtered through glass wool. In this way phialospore suspensions which were free of aleuriospores and hyphal fragments were produced, although only in small quantities.

Ultraviolet irradiation. Aleuriospores were used for u.v. irradiation rather than phialospores, despite their multinucleate nature, because of the difficulty of obtaining large numbers of phialospores and their poor germination (< 1 %). Ten ml of an aleuriospore suspension (5×10^6 /ml) in 0.1 M-phosphate buffer pH 6.0 were transferred to a 9 cm diameter Petri dish containing a sterile paper clip. This was placed on a magnetic stirrer under a Hanovia Chromalite u.v. lamp (80 % output at 254 nm wavelength) arranged so that the lamp was 5 cm above the surface of the spore suspension. Samples (0.3 ml) were collected at various time intervals up to 60 min and plated on to CMA.

Isolation and characterization of auxotrophic mutants. Survivors from u.v. irradiation were transferred to fresh CMA plates and replicated on to MMA. Suspected auxotrophic mutants were tested on MMA supplemented with (i) 3 mg Casamino acids/ml, (ii) 2 % (v/v) vitamin solution and (iii) a mixture of adenine, thymine, cytosine and guanine, each at 0.1 mg/ml. According to their response to these complex supplements the mutants were then tested on MMA with single supplements at the following final concentrations: amino

acids, 0.1 mg/ml; biotin, 1 µg/ml; riboflavin, 10 µg/ml; other vitamins, 2 µg/ml; adenine, thymine, cytosine and guanine, 0.1 mg/ml.

Mixed cultures. Four methods were used in attempting to produce prototrophic mycelia from mixed cultures of auxotrophic mutants on MMA: (A) two mutants were inoculated side by side on CMA, incubated for 3 days, and then hyphae from the interface were transferred to MMA; (B) mass hyphal inocula of two mutants were placed side by side on MMA and macerated together with glass needles; (C) mycelia from two mutants were macerated in Tween 80, mixed, and the mixture plated on MMA; (D) aleuriospore suspensions of two mutants were mixed in CM, incubated overnight, and then 0.1 ml portions plated on to MMA. With all four methods the mixed inocula were incubated for up to 15 days and examined periodically.

Treatment with p-fluorophenylalanine. A 0.1 % (w/v) solution of *p*-fluorophenylalanine (FPA), prepared by the method of Day & Jones (1969), was added to CMA to give final concentrations of 60, 80, 100, 120, 140 and 160 µg/ml. The wild-type 20-31, its auxotrophic mutants and the derived prototrophic strains were point-inoculated in replicate on to these FPA media and incubated for 10 days. Vigorously growing sectors arising from the prototrophic strains at FPA concentrations of 140 and 160 µg/ml were isolated and scored for auxotrophic and morphological markers. Not more than one sector was isolated from any single colony.

Determination of nuclear size. Strains were grown in slide cultures (Riddel, 1950) for 5 days, after which mycelia and spores adhering to the coverslips were fixed in acetic-alcohol (1:3, v/v) for 10 min and stained with Giemsa following the method of Robinow & Caten (1969). Suitably stained phialospore chains were photographed and the diameters of 20 phialospore nuclei were measured for each strain from prints ($\times 8000$ final magnification) using calipers.

RESULTS

Induction of mutants

The dose-response relationship for the lethal effect of u.v. irradiation on aleuriospores of strain 20-31 (Fig. 1) clearly possesses the shoulder expected with multinucleate spores (Atwood & Norman, 1949). Up to 10 min exposure, no reduction in spore viability was observed. After 10 min, survival decreased linearly on a logarithmic scale, falling to approximately 1 % after 50 min. The segregation of single aleuriospore cultures of 20-31 into black and white colony types was apparent among the survivors of u.v. irradiation. The proportion of white cultures increased with increasing dose, suggesting that potentially 'white' aleuriospores were more resistant. This relationship will be considered in detail in a subsequent publication.

Altogether, 450 black and 450 white survivors were sampled from the 45 min (2.3 % survival) and 60 min (0.5 % survival) treatments and tested for nutritional requirements. Four non-leaky auxotrophic mutants were found (Table 1). Aleuriospores from a black (20-31B) and a white (20-31W) segregant were similarly treated and from 300 survivors of each only one further auxotroph was recovered (Table 1). All five of these auxotrophic mutants were stable, no prototrophic revertants being recovered among 1.5×10^7 aleuriospores of each plated on MMA.

Mixed cultures of auxotrophs

Vigorously growing colonies arose on MMA from mixed cultures of the auxotrophic mutants established by methods B, C and D, but not by method A. No such prototrophic

Fig. 1. Lethal effect of u.v. irradiation (254 nm) on aleuriospores of *Humicola* sp. strain 20-31.

Table 1. *Characteristics of auxotrophic mutants induced in Humicola strains 20-31 and 20-31W by u.v. irradiation*

Mutant no.	Phenotype		Genotype	Parent strain
	Colour	Nutritional requirement		
M4	Black	Lysine	<i>whi⁺ lysA4</i>	20-31
M16	Black	Pyridoxine	<i>whi⁺ pdxA16</i>	20-31
M17	White	Arginine	<i>whiA1 argA17</i>	20-31
M18	White	Methionine	<i>whiA1 metA18</i>	20-31
M11	White	Pyridoxine	<i>whiA1 pdx-11</i>	20-31W

colonies arose in comparable inoculations of the mutant strains singly on to MMA. Altogether, seven different combinations of the five mutants were tested (Table 2) and prototrophs were recovered in each. Quantification of the frequency of prototroph formation was difficult except with method B, where after 15 days' incubation from 0.5 to 2.5 % of mixed cultures had given rise to vigorously growing mycelia, depending upon the particular combination (Table 2). A stock culture of the prototrophic mycelium from each combination was established by single hyphal tip transfer. These cultures will be referred to as the prototrophic strains and were used for all subsequent studies.

All prototrophic strains produced from a combination of a black and a white auxotroph were black. Combinations M17+M18 and M4+M16, which involved two white and two black auxotrophs respectively, gave white and black prototrophs respectively. The rate of increase in colony diameter on MMA of the prototrophic strains ranged from 56 to 111 % of that of wild-type 20-31, depending upon the particular combination. The component mutants grown singly failed to grow at all on MMA (Table 2). The variation in growth rate among the combinations is significant, reflecting differential pleiotropic effects of the mutations on growth.

Table 2. Frequency of prototrophic strains arising from mixed culture of auxotrophic mutants of *Humicola* strain 20-31 and the increase in colony diameter on MMA of these prototrophs and their parents

Mass hyphal inocula of two mutants were placed side by side on MMA and macerated together with glass needles (method B). The increases in colony diameter shown are the means of three replicates of each strain.

Strains	Percentage of cultures giving prototrophs*	Increase in colony diameter (mm/day)
Controls		
20-31	100	3.6
M4	0	0
M11	0	0
M16	0	0
M17	0	0
M18	0	0
Mixed cultures		
M4 + M11	2.5	3.1
M4 + M16	1.5	3.1
M4 + M17	1.0	2.9
M4 + M18	1.0	2.1
M16 + M17	1.0	4.0
M16 + M18	0.5	3.1
M17 + M18	0.5	2.0

* Two hundred cultures tested in each case.

Propagation of prototrophic strains

Initially it was suspected that the prototrophic mycelia arising from the mixed cultures were heterokaryons. To test this they were propagated on to CMA by macerated hyphae, single hyphal tips, single aleuriospores and single phialospores, and the resultant cultures tested for growth on MMA and on MMA with appropriate supplements. Heterokaryons should be resolved into their component homokaryons by these treatments with a frequency related to the method of propagation, being highest (100 %) for the uninucleate phialospores and lowest for hyphal tips and macerated hyphae. Segregation of the parental markers was not observed, however, and all subcultures retained both the nutritional and colour characteristics of the prototrophic strain from which they were derived, irrespective of the method of propagation (Table 3).

Spore and nuclear size of prototrophic strains

The stability of the prototrophic strains suggested that they might be diploids rather than heterokaryons. Spore size has frequently been used to distinguish diploid from haploid strains (see Tinline & MacNeil, 1969, for review). Aleuriospores of the present strains were very variable in size and no differences were apparent between any strains. Similarly, phialospores proved to be variable and, although on average those from the prototrophic strains were larger than those from strain 20-31 or its auxotrophic derivatives (Fig. 2), there was considerable overlap. Clutterbuck & Roper (1966) showed that haploid and diploid strains of *Aspergillus nidulans* differed in nuclear size. Measurements of nuclear size were made on phialospores from the prototrophic strains and the wild-type strains 20-31, 20-31W and 20-31B. Phialospores were selected because their nuclei are more regular in shape than hyphal nuclei while those of aleuriospores are difficult to stain through the thick spore wall (de Bertoldi, 1972). Nuclear diameters of the seven prototrophic strains

Table 3. *Nutritional characteristics of subcultures established by four different methods of propagation of prototrophic strains formed by mixed culture of auxotrophic mutants of Humicola strain 20-31*

Subcultures were established on CMA by plating macerated hyphae, single aleuriospores or single phialospores and by single hyphal tip transfers, and tested for their nutritional requirements by transfer to MMA and MMA with appropriate supplements.

Subcultures established by:	No. of prototrophic strains tested	No. of subcultures*	
		Prototrophic	Auxotrophic
Macerated hyphae	4	200	0
Single hyphal tips	7	350	0
Single aleuriospores	3	150	0
Single phialospores	6	300	0

* Fifty subcultures were established from each strain for each method of subculture.

Fig. 2. Phialospore chains of (a) 20-31 and (b) M4+M17 stained with Giemsa. Note the larger size of the spores and nuclei in (b) than in (a).

Table 4. Nuclear diameter for original parental strains and derived prototrophic strains of *Humicola* strain 20-31

The diameters of 20 phialospore nuclei were measured for each strain from photographic prints of Giemsa-stained preparations.

Type of strain	Strain no.	Nuclear diameter (μm)		Mean nuclear diameter for strain type (μm)
		Mean	Range	
Parental	20-31	0.51	0.46-0.56	0.51 $\pm$ 0.006
	20-31W	0.51	0.45-0.55	
	20-31B	0.51	0.40-0.56	
Prototrophic	M4+M11	0.64	0.58-0.71	0.64 $\pm$ 0.004
	M4+M16	0.64	0.53-0.75	
	M4+M17	0.66	0.61-0.70	
	M4+M18	0.63	0.58-0.71	
	M16+M18	0.64	0.55-0.70	
	M17+M18	0.64	0.61-0.67	

were consistently larger than those of the wild-type strains and there was almost no overlap in the distributions (Table 4 and Fig. 2). The ratio of the mean nuclear diameter of prototrophic to wild-type strains is 1.25:1. This corresponds to a difference in nuclear volume of 1.97:1, assuming the nuclei are spherical.

Marker segregation from prototrophic strains

The comparisons of nuclear size suggested that the prototrophic mycelia arising in mixed cultures of auxotrophs were diploid. To test this, attempts were made to haploidize the suspected heterozygous diploids and obtain segregation of the markers by treatment with FPA (Morpurgo, 1961; Lhoas, 1961). Prototrophic strains showed increasing inhibition when grown on CMA containing FPA at 60, 80, 100, 120, 140 and 160 $\mu\text{g}/\text{ml}$. The wild-type 20-31 and its auxotrophic derivatives were less inhibited at all concentrations. At 140 and 160 μg FPA/ml the prototrophic strains, but not the wild-type or auxotrophic mutants, produced more vigorously growing sectors. Twenty-five sectors were isolated from each of the seven prototrophic strains and tested for segregation of colour and auxotrophic markers. With each strain segregation was observed for the two or three markers suspected to be heterozygous (Table 5). Segregation was consistent with a 1:1 ratio in 15 out of the total of 19 single allele pairs tested (considering each prototrophic strain separately). Three of the four significant deviations resulted from an excess of the wild-type allele. The excess of the mutant allele, *pdxA*, for combination M16+M17 may be explained by the association of *pdxA* with *argA*⁺, the latter allele being recovered in excess. With the exception of *pdxA* and *argA* all the markers segregated independently, although the frequencies of recovery of the different genotypes from any one prototrophic strain were often heterogeneous (Table 5).

When FPA-induced segregants, including prototrophs, were reinoculated on to FPA media they possessed the higher tolerance characteristic of 20-31 and its original auxotrophic derivatives and did not produce more vigorous sectors or further marker segregation. Nuclear diameters were measured for eight prototrophic segregants originating from three different prototrophic strains. All had nuclei similar in size to 20-31 and smaller than the prototrophic strains from which they were derived.

Table 5. *Genotypes and their frequencies recovered among FPA-induced segregants from seven prototrophic strains of Humicola strain 20-31*

Twenty-five vigorously growing sectors were isolated from each of seven prototrophic strains growing on CMA containing FPA at 140 or 160 µg/ml and classified for the colour and nutritional markers which differentiated their parent mutant strains.

Prototrophic strain	Genotype of mutant parents*		Number of segregants of genotype†											
	A	B	+	+	+	+	+	+	+	+	+	+	+	+
M4 + M16	<i>whiA</i> ⁺ <i>lysA</i> <i>pdxA</i> ⁺	<i>whiA</i> ⁺ <i>lysA</i> ⁺ <i>pdxA</i>	9	5	6	5	—	—	—	—	—	—	—	—
M4 + M17	<i>whiA</i> ⁺ <i>lysA</i> <i>argA</i> ⁺	<i>whiA</i> <i>lysA</i> ⁺ <i>argA</i>	3	3	4	3	5	2	2	2	2	2	3	3
M4 + M18	<i>whiA</i> ⁺ <i>lysA</i> <i>metA</i> ⁺	<i>whiA</i> <i>lysA</i> ⁺ <i>metA</i>	6	2	2	3	5	4	4	2	2	1	1	1
M16 + M17	<i>whiA</i> ⁺ <i>pdxA</i> <i>argA</i> ⁺	<i>whiA</i> <i>pdxA</i> ⁺ <i>argA</i>	0	4	4	0	0	0	1	16	0	0	0	0
M16 + M18	<i>whiA</i> ⁺ <i>pdxA</i> <i>metA</i> ⁺	<i>whiA</i> <i>pdxA</i> ⁺ <i>metA</i>	4	3	8	2	0	4	4	1	3	3	4	4
M17 + M18	<i>whiA</i> <i>argA</i> <i>metA</i> ⁺	<i>whiA</i> <i>argA</i> ⁺ <i>metA</i>	—	—	—	—	10	3	8	1	4	4	4	0
M4 + M11	<i>whiA</i> ⁺ <i>lysA</i> <i>pdx</i> [—]	<i>whiA</i> <i>lysA</i> ⁺ <i>pdx</i> —	12	3	5	2	0	2	1	1	1	1	1	0

* A and B correspond to the two mutant parent strains for each prototrophic combination respectively; e.g. for M4 + M16, A = M4 and B = M16.

† The three symbols correspond to the three markers in the order shown in the parental genotypes. —, Genotype not expected.

DISCUSSION

Three lines of evidence indicate that the prototrophic strains which arise directly from mixed cultures of auxotrophic mutants of the *Humicola* strain 20-31 are heterozygous diploids. Firstly, they remain stably prototrophic and do not segregate when propagated by single protoplasmic units, including uninucleate phialospores. Secondly, they contain nuclei of approximately twice the volume of those of strain 20-31. Thirdly, they segregate for markers distinguishing their immediate parent auxotrophic strains. Similarly, several related observations indicate that the segregants recovered from FPA treatment of these prototrophic diploid strains are haploid. These are: (i) the known haploidizing effect of FPA on somatic diploids in other fungal species (Morpurgo, 1961; Lhoas, 1961; McCully & Forbes, 1965); (ii) the simultaneous expression of the various auxotrophic requirements, the latter being invariably determined by recessive alleles; (iii) the failure to induce segregation in first order segregants, including prototrophs, by a second FPA treatment; and (iv) the reduction in nuclear size following FPA treatment. Taken together these observations demonstrate that auxotrophic mutant derivatives of strain 20-31 readily form somatic heterozygous diploids and that the latter can be induced to haploidize by treatment with FPA. Thus, two of the steps of the parasexual cycle (Pontecorvo, 1956; Roper, 1966) have been demonstrated in this strain. It remains to be established whether mitotic crossing-over also occurs.

An unusual feature of the present results is the isolation of heterozygous diploid strains from mixed cultures of auxotrophic mutants without a detectable, intervening, heterokaryotic state. Hyphal anastomosis and heterokaryon formation would appear essential prerequisites for the formation of somatic heterozygous diploids in filamentous fungi (Pontecorvo, 1956; Roper, 1966). Extensive, freely growing heterokaryons, such as are produced by many *Aspergillus* spp., are not essential for diploid formation however. In *Penicillium chrysogenum* (Pontecorvo & Sermoniti, 1954) and *Cephalosporium acremonium* (Nuesch, Treichler & Liersch, 1973), where heterokaryons grow poorly, diploids arise directly as vigorously growing sectors from the heterokaryotic mycelium. The *Humicola* strain studied would appear to be an extreme example of this phenomenon, although it is possible that microscopic examination of the mixed cultures would reveal a restricted heterokaryon which is swamped by the vigorous diploid mycelium once this is formed. That diploids can be formed without growth of an intervening heterokaryon is shown by their ready isolation from fused sporidia in *Ustilago violacea*, where the dikaryons cannot grow in culture (Day & Jones, 1968).

The demonstration of heterozygous diploid formation and haploidization makes genetic analysis possible in this imperfect soil fungus. The morphological stability of the black/white diploids and their clear segregation on haploidization into the two parental types with no intermediates suggests that the difference between the spontaneous black and white segregants is under chromosomal control, and that probably only a single gene (*whiA*) is involved (the possibility of a group of genes on the same linkage group cannot be excluded). This contrasts with the situation found by Hastie (1962) in *Verticillium albo-atrum*, where a similar, naturally occurring difference in pigmentation appeared to be cytoplasmically determined. Assuming that mitotic crossing-over is rare and that most reassorted genotypes present in the FPA-induced segregants arise through independent chromosome assortment during haploidization, assumptions which hold in other fungal species (Käfer, 1961; McCully & Forbes, 1965; Lhoas, 1967; Day & Jones, 1968), we can, from the data in Table 5, assign the markers studied to at least four linkage groups as follows: I, *whiA*;

II, *lysA*; III, *pdxA argA*; IV, *metA*. The remaining marker, *pdx-*, segregated independently of *whiA* and *lysA* and therefore is not in linkage groups I or II. It was not tested against the group III or IV markers or for allelism with *pdxA*, however, and therefore may either be in one of these or represent a fifth linkage group.

The simple genetic differentiation of the black and white strains demonstrates that this very obvious character does not reflect extensive divergence and hence should not be given much weight in any classification. Parasexual genetic analysis may now be applied to other natural character differences and instances of spontaneous variation, in an attempt to determine their origins and judge their potential taxonomic significance. In addition to these indirect aids to classification, investigation of the formation and properties of heterozygous diploids may be of value for the study of homologies in imperfect fungi. For example, Hastie (1973) has shown in *Verticillium* spp. that recombination is less in interspecific diploids than in intraspecific diploids. However, the widespread occurrence within species of filamentous fungi of simply inherited, vegetative (heterokaryon) incompatibility systems (Caten & Jinks, 1966; Caten, 1972) means that the failure to isolate diploids should be interpreted with caution.

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