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Further studies on the genetics of *Cephalosporium acremonium* using protoplast fusion methods

G. Perez Martinez and J.F. Peberdy

Department of Botany, Microbial Biochemistry and Genetics Group, University of Nottingham, Nottingham NG7 2RD, U.K.

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SUMMARY

Genetic analysis of protoplast fusants in *Cephalosporium acremonium* can be carried out by two complementary approaches: analysis of stable haploid segregants and analysis of unstable heterozygotes of uncertain ploidy. However, segregation may be distorted by physiological as well as genetic phenomena, i.e., cross-feeding, syntrophic growth, allele viability, clonal effects, or parental genome segregation.

INTRODUCTION

Parasexual crosses in *Cephalosporium acremonium* have been attempted in the past, with reports describing the recovery of weakly growing heterokaryotic colonies [6,11,12] and the isolation of a single heterozygous diploid [12]. Haploid segregants were occasionally recovered directly from heterokaryons [3,11,12]. Using protoplast fusion methods, however, progeny could be recovered on selective regeneration media [2] and later Hamlyn and Ball [6] showed these to be either stable haploid segregants or unstable heterozygotes. The conclusion drawn from these observations by the various authors was that the heterokaryon and diploid appeared to be very unstable or transient in *C. acremonium*.

Subsequently a number of new protoplast-fusion crosses were carried out in this laboratory [5] and two forms of genetic analysis in *C. acremonium* were devised. In the first, polyethylene glycol (PEG)-treated protoplasts were plated on different selective media and stable haploid colonies that developed were subsequently analysed for their phenotypes. The second method involved the isolation of slow growing heterozygotes from selective fusion plates, their purification, and phenotypic analysis of haploid segregants which arose from them as spontaneous events. A preliminary linkage map was established from these data (Fig. 1).

The aim of the present study was to complement the linkage data from previous work and to indicate some of the difficulties involved in the parasexual analysis of this fungus.

Correspondence: Dr. J.F. Peberdy, Department of Botany, Microbial Biochemistry and Genetics Group, University of Nottingham, Nottingham NG7 2RD, U.K.

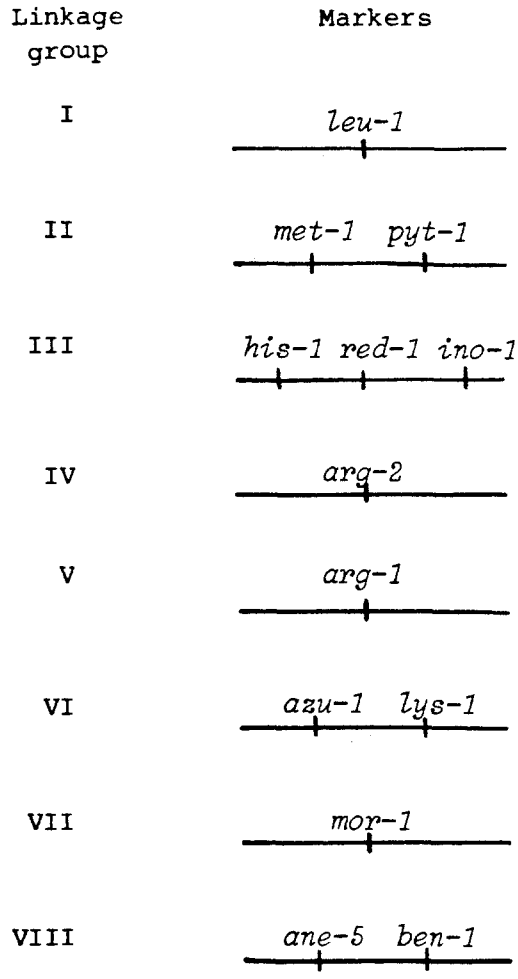

Fig. 1. Preliminary linkage map for *C. acremonium*

MATERIALS AND METHODS

Strains

All the mutant strains used were derived from *Cephalosporium acremonium* strain M8650, obtained from Glaxochem Ltd. Recombinant strains obtained from previous crosses carried out in this laboratory were also used [5]. A summary of the strains used and their origin is given in Table 1.

Medium composition

Strains were maintained on Sabouraud's complete medium [7]. Minimal medium (MM) was a modification of Czapek Dox medium as described by Hamlyn et al. [7]. For protoplast cultures 0.7 M KCl was added to media to provide osmotic support. The partial and totally supplemented media used to recover fusion products, control platings and phenotype analysis contained 0.1 g · l^{-1} of the required amino acids and inositol.

Protoplast isolation and fusion

The procedure used for protoplast isolation and fusion has been described in earlier reports [1,4,5,7,8].

Selection of fusion products

The conditions used for regeneration of protoplasts and selection of fusion products on nutritionally deficient media have been described in previous work [5,7]. Supplementation of MM was so

Table 1

Collection number, genetic markers and origin of the strains used for protoplast fusion crosses

Markers: *arg-1, arg-2, arg-6* – requirement for arginine; *ane-5* – requirement for aneurine; *azu-1* – resistance to azuracil; *ben-1* – resistance to benzimidazole; *ino-1* – requirement for inositol; *leu-1, leu-3* – requirement for leucine; *lys-1* – requirement for lysine; *met-1* – requirement for methionine; *mor-1* – altered colony morphology; *phe-1* – requirement for phenylalanine; *pyt-1, pyt-2* – resistance to pyrathiamine; *red-1* – red colony pigment.

Collection number	Origin	Genetic markers
20–4	UV mutagenesis	*red-1, arg-1, leu-1, azu-1, ben-1, pyt-1, mor-1*
20–5	UV mutagenesis	*arg-2, azu-1, met-1*
20–8	UV mutagenesis	*ane-5, ino-1*
20–12	UV mutagenesis	*arg-6, lys-1, leu-3, pyt-2*
20–13	UV mutagenesis	*lys-1, phe-1*
20–20	Recombination of 20-4 × 20-8	*ino-1, leu-1, ben-1, mor-1*

designed to select only recombinant fusion progeny.

Controls were also plated at different stages of the fusion experiments. These were designed to establish the forward mutation frequency of each marker, the presence of hyphal fragments, the occurrence of cross-feeding between the protoplasts of the parental strains and the survival of protoplasts after PEG treatment.

Analysis of crosses

Colonies recovered from fusion plates range from stable haploids to unstable heterozygotes. The first type are large colonies with non-parental phenotype and are stable in the presence of haploidizing agents [5]. The heterozygote colonies are smaller and compact, and under normal conditions spontaneously segregate to give progeny with parental and segregant phenotypes. Thus two approaches to genetic analysis can be performed after protoplast fusion according to the stage of segregation chosen.

Individual colonies were inoculated onto the relevant diagnostic media, where the selective and non-selective markers including mycelial pigmentation and resistance to growth inhibitors were scored. Pairwise arrangement for the allelic combinations was used and the statistical significance of linkages was determined by a 2×2 contingency test.

RESULTS

Cross-feeding and 'leaky' markers

Two problems were encountered during the culture of protoplast fusion products: the syntrophic growth of parental hyphae with the formation of cross-feeding colonies and the occurrence of 'leaky' markers in some of the parental strains. The first phenomenon resulted in colonies of normal size with a soft texture, from which only the parental phenotypes could be recovered when they were fragmented and plated on complete medium. Leakiness of one of the parental markers produced a rich background growth that made it difficult to isolate fusion products from the selective medium. In some strains the problem was accentuated in the protoplast 'state'.

Linkage studies

Analysis of heterozygote segregants proved to be as useful as the analysis of haploid segregants for the establishment of linkage relationship in *C. acremonium*. Heterozygote analysis is theoretically possible for every cross, whereas haploid segregant analysis is sometimes technically difficult for the reasons mentioned above.

Cross 1: 20-5 (arg-2, azu-1, met-1) × 20-20 (ino-1, leu-1, ben-1, mor-1)

This cross highlights the problem where the 'leaky' marker *met-1* was used and cross-feeding occurred. Only one selective medium (MM + methionine + inositol) could be used and analysis of the haploid recombinants showed free assortment of the parental markers *met-1, ino-1, arg-2, azu-1* and *ben-1* which were known to be located on distinct linkage groups (Fig. 1). Clearly the use of this selective medium prevents the growth of arg^- and leu^- phenotypes and therefore any linkage analysis. The analysis of phenotypes derived from a heterozygote from this cross is shown in Table 2. With the exception of *Arg*, *Leu* and *Met* the markers showed roughly equal segregation. In the case of *Leu* all the progeny were leu^+ and for *Arg* and *Met* there was an excess of arg^- and met^- phenotypes (Table 2a and b).

Cross 2: 20-4 (red-1, arg-1, leu-1, azu-1, ben-1, pyt-1, mor-1) × 20-12 (arg-6, leu-3, lys-1, pyt-2)

Five selective markers were involved in cross 2 (Table 3a–c) but both parental strains shared two requirements, arginine and leucine, which, were obtained from separate mutagenic treatments. The *arg-1* marker in strain 20-4 caused a block in the urea cycle before ornithine and citrulline, whereas strain 20-12 carrying *arg-6* could not utilize either intermediate in place of arginine. The biochemistry of *leu-1* and *leu-3* could not be resolved because the required intermediates were not available and the segregation of these two markers could not be followed. Initially, therefore, the two mutations for both requirements appeared mutually complementary, *arg-1* with *arg-6* and *leu-1* with *leu-3*, for prototrophy was detected in a number of isolates.

Table 2

(a) Genetic analysis of haploid segregants recovered on selective media in cross 1: 20-5 × 20-20 (*arg-2, azu-1, met-1* × *ino-1, ben-1, leu-1, mor-1*)

Only colonies developing on MM supplemented with methionine and inositol could be used for genetic analysis, due to cross-feeding and leakiness of *met-1*. Figures that are underlined denote recombinants. A positive sign represents the wild type allele and a negative sign the mutant.

		met-1 +	*met-1* −	*ino-1* +	*ino-1* −	*azu-1* +	*azu-1* −
ben-1	+	33	1	26	8	32	2
	−	43	1	19	25	43	1
azu-1	+	73	2	42	33		
	−	3	0	3	0		
ino-1	+	45	0				
	−	31	2				

(b) Phenotypes and pairwise table of the segregants from a heterozygote in cross 1

Phenotypes

arg-2	*leu-1*	*met-1*	*ino-1*	*azu-1*	*ben-1*	No. segregants
−	+	−	−	−	−	5
−	+	−	−	+	−	6
−	+	−	+	−	−	9
−	+	−	+	+	−	3
−	+	−	+	−	+	9
−	+	−	+	+	+	3
−	+	−	−	−	+	16
−	+	−	−	+	+	14
−	+	+	+	−	+	1
−	+	+	+	+	+	3
−	+	+	−	+	−	3
−	+	+	+	+	−	3
+	+	+	−	−	+	1
+	+	+	+	−	+	2
−	+	+	−	+	+	1
						79

Genetic Analysis

		arg-2 +	*arg-2* −	*met-1* +	*met-1* −	*ino-1* +	*ino-1* −	*azu-1* +	*azu-1* −
ben-1	+	3	47	8	42	18	32	21	29
	−	0	29	6	23	15	14	15	14
azu-1	+	0	36	10	26	12	24		
	−	3	40	1	39	21	22		
ino-1	+	2	21	9	24				
	−	1	45	5	41				
met-1	+	3	11						
	−	0	65						

If genetically unlinked to other markers, the expected frequencies of the alleles leu^+:leu^- and arg^+:arg^- under non-selective conditions should approach the ratio 1:3. On the two selective media supplemented with arginine (MM + lysine + arginine and MM + arginine) the observed ratios of arg^+:arg^- were 25:95 and 41:113, the expected ratios being 30:90 and 38.5:115.5, respectively. Non-linkage of *arg-1* and *arg-6* to other markers involved in the cross is therefore clear. On the contrary, the frequencies observed for leu^+: leu^- on the selective medium supplemented with lysine and leucine (MM + lysine + leucine), were significantly different from the expected segregation (1:3). Analysis of segregants recovered from MM + lysine + leucine suggests apparent linkage of *lys-1* with *red-1*, *azu-1* and *ben-1*. The same phenomenon may also be the cause of apparent loss of linkage observed between *lys-1* and *azu-1* on the selective medium containing lysine and arginine. Analysis of a heterozygote in the same cross (Table 3c), revealed the complementation of *arg-1* and *arg-6*, and *leu-1* and *leu-3*.

As is shown by Table 3b, certain phenotypes are recovered with a much higher frequency. Thus certain unlinked markers do not appear to be reas-

Table 3

(a) Combination of markers found on the different selective media in cross 2, and the number of markers recombined in each phenotype with respect to the parentals

red-1	*lys-1*	(*leu*)	(*arg*)	*azu-1*	*ben-1*	Medium			Parental markers	
						MM + Lys + Leu	MM + Lys + Arg	MM + Arg	20-12	20-4
–	+	–	–	–	–	/	/	/(P)	4	0
–	+	–	+	–	–	33	/	/	5	1
–	+	+	–	–	–	/	77	103	5	1
–	+	+	+	–	–	3	14	16	6	2
+	+	+	+	–	–	4	5	24	5	3
–	–	+	–	–	–	/	8	/	4	2
+	–	+	–	–	–	/	2	/	3	3
–	–	+	–	–	–	/	12	/	4	2
–	+	+	+	+	–	0	1	0	5	3
+	+	–	+	–	–	1	/	/	4	2
–	+	+	–	+	–	/	0	1	4	2
+	+	+	–	–	–	/	1	2	4	2
+	–	–	–	+	+	/	/	/(P)	0	4
+	–	–	+	+	+	82	/	/	1	5
+	–	+	–	+	+	/	1	/	1	5
+	–	+	+	+	+	/	1	/	2	6
–	–	+	–	+	+	/	1	/	2	4
–	–	–	+	+	+	2	/	/	2	4
+	+	+	–	+	+	0	0	1	2	5
+	–	+	+	+	–	1	0	/	3	5
+	–	–	+	–	+	1	/	/	2	4
–	+	+	+	+	+	1	1	0	4	4
+	+	+	–	+	–	/	0	2	3	3
+	+	+	+	+	–	0	2	0	4	4
+	–	+	+	–	–	/	1	/	4	4
						128	127	149		

(b) Genetic analysis of haploid recombinants recovered from selective media in cross 2: 20-4 × 20-12 (*red-1*, *arg-1*, *leu-1*, *azu-1*, *ben-1*, *pyt-1*, *mor-1*[b] × *arg-6*, *leu-3*, *lys-1*, *pyt-2*)

Figures that are underlined denote recombinants. Positive and negative signs represent the wild type and mutant alleles, respectively.

MM + lysine + leucine[a]

		red-1		*lys-1*		(*leu*)[c]		*azu-1*	
		+	–	+	–	+	–	+	–
ben-1	+	83	4*	1*	86	1*	86	86	1*
	–	5	36	40	1	7	34	1	40
azu-1	+	83	4*	1*	86	2	85		
	–	5	36	40	1	6	35		
(*leu*)[c]	+	4	4	7	1*				
	–	84	36	34	86				
lys-1	+	4*	37						
	–	84	3						

Table 3b, contd.
MM + lysine + arginine

		red-1		*lys-1*		*arg-1*		*arg-6*	*azu-1*	
		+	−	+	−	+	−	−	+	−
ben-1	+	2	2	1	3	2	2	0	4	0
	−	12	104	101	15	23	80	13	3	113
azu-1	+	4	3	4[d]	3	5	2	0		
	−	10	103	98	15[d]	20	80	13		
arg	+	9	16	23	2	**				
arg-1	−	4	78	66	16	**				
arg-6		1	12	13	0	**				
lys-1	+	9	93							
	−	5	12							

MM + arginine

		red-1		(*arg*)[e]		*azu-1*	
		+	−	+	−	+	−
ben-1	+	1	1	1	1	2	0*
	−	28	124	40	112	3*	149
azu-1	+	3	2	1	4		
	−	26	123	40	109		
(*arg*)[e]	+	25	16				
		4	109				

* Free rearrangement, $P < 0.005$
** χ^2 test for phenotype frequency 1:3
[a] Cross-feeding was found on MM supplemented with leucine.
[b] *mor-1* was not scored.
[c] Mutations *leu-3* and *leu-1* could not be resolved.
[d] χ^2 test, linkage not significant.
[e] All *arg*$^-$ phenotypes corresponded with *arg-1*.

(c) Phenotypes and pairwise tables of the segregants from a heterozygote in cross 2

Phenotypes

red-1	(*arg*)	*lys-1*	(*leu*)	*ben-1*	*azu-1*	*pyt-1*	(*mor*)	No. segregants
+	+	+	+	−	−	−	0	7
+	−	−	−	+	+	−	0	4
+	−	−	−	+	+	−	1	6
+	−	−	−	+	+	−	2	3
+	+	−	+	−	+	−	1	1
+	+	−	+	−	+	−	0	1
−	−	+	+	−	−	−	1	1
+	−	−	+	−	+	−	1	2
+	−	+	+	−	−	−	0	4
+	+	+	+	−	−	−	1	1
+	+	+	−	+	+	−	1	1

Table 3c, contd.
Genetic analysis

		red-1 +	*red-1* −	(*arg*)[a] +	(*arg*)[a] −	*lys-1* +	*lys-1* −	(*leu*) +	(*leu*) −	*ben-1* +	*ben-1* −
azu-1	+	18	0	3	15	1*	17	4	14	14	4*
	−	12	1	8	5	13	0*	13	0	0*	13
ben-1	+	14	0	1	13	1*	13	0	4		
	−	1	1	10	7	13	4*	17	0		
(*leu*)	+	16	0	10	7	13	4				
	−	16	1	1	13	1	13				
lys-1	+	13	1	9	5						
	−	17	0	2	15						
(*arg*)	+	11	0								
	−	19	1								

* Free arrangement $P < 0.005$.
[a] *arg* and *leu* markers were not resolved.

sorted at random, probably due to chromosomal rearrangements introduced in the parental strains during their isolation with UV radiation.

Both parental strains also carried mutations conferring resistance to pyrithiamine identified as *pyt-1* and *pyt-2*. These two mutations showed no complementation on any of the selective media.

The morphological variant described as *mor-1* had been introduced together with *arg-1* marker by UV mutagenesis [5]. The segregation of this marker was followed on all four selective media used in cross 3 (Table 4), showing independent segregation from the rest of the markers involved, known to be positioned on linkage groups I, II, III, V, VI and VIII.

Cross 3: 20-4 (red-1, arg-1, leu-1, azu-1, ben-1, pyt-1, mor-1) × 20-13 lys-1, phe-1)

A very low recovery of the *phe* segregants in this cross (Table 4a) could be interpreted as an apparent linkage of *phe-1* to *lys-1* and *azu-1*. However, *phe-1* proved to be unlinked to azu-1. The remainder of the markers involved in this cross showed a random assortment. Linkage of *azu-1*, *lys-1* was confirmed and the analysis suggests that *phe-1* is not linked to any of the known markers.

A pronounced clonal effect was found in the segregants of the heterozygote derived from this cross (Table 4b). Thirty-five of the 52 sectors analysed showed the same phenotype. The *ben-1* gene segregated together with *lys-1* and *azu-1*, and *phe-1* showed independent segregation from the other markers.

Table 4

(a) Genetic analysis of recombinants from selective media in cross 3: 20–4 × 20–13 (*red-1*, *arg-1*, *leu-1*, *azu-1*, *ben-1*, *pyt-1*, *mor-1* × *lys-1*, *phe-1*)

Figures that are underlined denote recombinants. Positive and negative signs represent the wild type and mutant alleles, respectively.

MM + lysine + leucine

		red-1		*lys-1*		*leu-1*		*azu-1*		*ben-1*		*pyt-1*	
		+	−	+	−	+	−	+	−	+	−	+	−
mor-1	+	45	9	22	32	44	10	32	22	34	20	38	16
	−	3	3	5	1	4	2	1	5	3	3	4	2
pyt-1	+	34	8	20	22	35	7	24	18	29	13		
	−	14	4	7	11	13	5	9	9	8	10		
ben-1	+	32	5	15	22	28	9	21	16				
	−	16	7	12	11	20	3	12	11				
azu-1	+	29	4	2*	31	27	6						
	−	19	8	25	2*	21	6						
leu-1	+	30	9	21	27								
	−	9	3	6	6								
lys-1	+	17	10										
	−	31	2										

MM + lysine + arginine

		red-1		*lys-1*		*arg-1*		*azu-1*		*ben-1*		*pyt-1*	
		+	−	+	−	+	−	+	−	+	−	+	−
mor-1	+	45	17	59	3	37	25	3	59	38	24	33	29
	−	2	18	20	0	2	18	0	20	2	18	2	18
pyt-1	+	24	11	32	3	20	15	3	32	20	15		
	−	23	24	47	0	19	28	0	47	20	27		
ben-1	+	31	9	37	3	22	18	3	37				
	−	16	26	42	0	17	25	0	42				
azu-1	+	3	0	0*	3	3	0						
	−	44	35	79	0*	36	43						
arg-1	+	25	14	36	3								
	−	22	21	43	0								
lys-1	+	44	35										
	−	3	0										

MM + phenylalanine + leucine

		red-1		*leu-1*		*phe-1*		*azu-1*		*ben-1*		*pyt-1*	
		+	−	+	−	+	−	+	−	+	−	+	−
mor-1	+	64	23	83	4	82	5	9	78	38	49	55	32
	−	4	6	9	1	10	0	0	10	7	3	2	8
pyt-1	+	41	16	56	1	57	0	5	52	24	33		
	−	27	13	36	4	35	5	4	36	21	19		
ben-1	+	31	14	44	1	41	4	2	42				
	−	37	15	48	4	51	1	6	46				
azu-1	+	9	0	9	0	9	0						
	−	59	29	83	5	83	5						
phe-1	+	63	29	88	4								
	−	5	0	4	1								
leu-1	+	64	28										
	−	4	1										

MM + phenylalanine + arginine

		red-1		*leu-1*		*phe-1*		*azu-1*		*ben-1*		*pyt-1*	
		+	−	+	−	+	−	+	−	+	−	+	−
mor-1	+	56	20	50	26	72	4	2	74	34	42	44	32
	−	5	14	4	15	19	0	1	18	3	16	1	18
pyt-1	+	34	11	29	16	43	2	1	44	17	28		
	−	27	23	25	25	48	2	2	48	20	30		
ben-1	+	31	6	22	15	34	3	2	35				
	−	30	28	32	26	57	1	1	57				
azu-1	+	0	3	2	1	3	0						
	−	61	31	52	40	88	4						
phe-1	+	57	34	53	38								
	−	4	0	1	3								
arg-1	+	37	17										
	−	24	17										

* Free arrangement, $P < 0.005$.

Table 4, contd.
(b) Phenotypes and pairwise table of segregants from a heterozygote in cross 3

Phenotypes

arg-1	*leu-1*	*lys-1*	*phe-1*	*azu-1*	*pyt-1*	*ben-1*	*red-1*	No. isolates
−	−	−	+	+	−	+	+	35
−	−	−	+	+	−	+	−	1
−	−	−	+	+	+	+	+	1
+	−	+	+	−	−	−	+	2
+	−	+	+	−	−	−	−	1
+	−	−	+	+	−	+	+	2
+	+	+	+	−	−	−	+	7
+	+	+	+	−	−	−	−	1
+	−	−	−	+	+	+	+	1
+	+	−	+	+	−	−	+	1
								—
								52

Genetic Analysis

		arg-1		*leu-1*		*lys-1*		*phe-1*		*azu-1*		*pyt-1*		*ben-1*	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
red-1	+	13	36	8	41	9	40	48	1	40	9	2	47	39	10
	−	2	1	1	2	2	1	3	0	1	2	0	3	1	2
ben-1	+	3	37	0	40	2	38	39	1	40	0	2	38		
	−	12	0	9	3	9	3	12	0	1	11	0	12		
pyt-1	+	1	1	0	2	0	2	1	1	2	0				
	−	14	36	9	41	11	39	50	0	39	11				
azu-1	+	4	37	1	40	0	41	40	1						
	−	11	0	8	3	11	0	11	0						
phe-1	+	14	37	9	42	11	40								
	−	1	0	0	1	0	1								
lys-1	+	11	0	8	3										
	−	4	37	1	40										
leu-1	+	9	0												
	−	6	37												

DISCUSSION

Our experiments further demonstrate that *C. acremonium* yields either unstable heterozygotes or stable haploid segregants directly after protoplast fusion, suggesting that the heterokaryon and diploid stages develop very rapidly. Accordingly the diploid in this fungus was described as a transient stage [6].

During the analysis of the fusion products in this work a parallel analysis of heterozygote colonies has proved to be very useful in confirming the linkage data obtained from the recombinant isolates recovered directly on the selective media. However, there are still a number of difficulties to be overcome in heterozygote analysis. First, the very rapid haploidization that takes place in many crosses allows the recovery of only a few heterozygous colonies from each fusion experiment. Apparently the diploid and early hyperhaploids have no greater stability than the more reduced stages, until the haploid genome is reached, which then overtakes the growing colony. Under special circumstances some heterozygous colonies remain stable for a longer time, making their isolation and purification possible. Frequently heterozygotes do not display segregation of all the parental markers, suggesting that non-disjunction has already occurred, e.g., heterozygotes from cross 1. Secondly, and as a result of these events, the protocol for the isolation of sectors needs to be improved in order to avoid the clonal effect occasionally found in these experiments, e.g., cross 3.

Despite the general instability shown by heterozygotes, stable forms can be recovered at an adequate frequency (1–5%), and these provide the opportunity for a complete genetic analysis of a given fusion experiment to be undertaken exclusively using the large number of segregants obtained from their spontaneous haploidization. The frequency of recovery of heterozygotes can be raised when selective pressure relates to linked markers in *trans* position [7,13]. Practically all the recombinants recovered on MM + histidine had the morphological characteristics of heterozygotes, and up to two subculture steps were necessary to induce the haploidization (Perez Martinez and Peberdy, unpublished data).

Genetic analysis of the crosses carried out strongly supports the existence of the eight linkage groups reported for *C. acremonium* [7]. The allocation of new markers has been attempted. Thus *arg-6* and *phe-1* segregated independently from most of the linkage groups previously identified (I–VIII) [5,7]. However, their assignment to new linkage groups (IX or X) should be resolved through further crosses. The segregation of leu^-:leu^+ phenotypes was not clear enough to be resolved. In cross 2, the pyrithiamine-resistant mutations *pyt-1* and *pyt-2* were involved. The absence of recombination indicated that they were allelic, and therefore could be renamed as *pyt-A1* and *pyt-A2*, respectively. The clear parental segregation observed in this cross (Table 4b) could be the consequence of the chromosomal rearrangements introduced in the parental strains (20-4 and 20-12) during their isolation using UV mutagenesis.

Markers on linkage group VI were involved in crosses 2 and 3. Data from MM + lysine + arginine in cross 2 and the progeny of a heterozygote in cross 3 were not used in the analysis, because of the respective lack of linkage between *lys-1* and *azu-1* and clonal effects. Therefore a total of 644 isolates were analysed with respect to these two markers and only 24 of them showed evidence for mitotic recombination, giving a recombination frequency of 3.7%. Hamlyn et al. [7] reported frequencies of 3.4% to 9% in different crosses and our finding is within this range. Together these observations indicate that mitotic crossing-over occurs with a higher frequency in *C. acremonium* than in *Aspergillus nidulans* (0.03 per genome per division [9]), although it is not yet possible to calculate a mitotic recombination index [10,14] because of the technical difficulty posed by the determination of the proportion of diploid nuclei which undergo haploidization in *C. acremonium*.

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