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Haploidization Analysis in *Penicillium chrysogenum*

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SUMMARY

Results relevant to the problem of increasing penicillin yield in *Penicillium chrysogenum* by recombination through the parasexual cycle are discussed. Sister strains differing from each other in few mutational steps have been used to construct a map with three haploidization groups. By this approach it has been possible to overcome those barriers to recombination, principally chromosome rearrangements, which arise when non-sister strains are used. Selection against an allele has been demonstrated and the problem solved by use of *p*-fluorophenylalanine to induce haploidization. In addition, the selection of a morphologically stable strain from an unstable strain has been achieved and the genetic determinant for this instability allocated to a haploidization group.

INTRODUCTION

In the imperfect fungus *Penicillium chrysogenum* initial improvements for penicillin yield depended upon mutation and selection (Elander, 1966). Discovery of the parasexual cycle and its occurrence in asexual fungi offered hope of breeding for improved penicillin yield (Pontecorvo & Sermonti, 1954; Pontecorvo, 1956). In the first attempts (Sermonti, 1959) the only strain with improved titre was a diploid. Macdonald and co-workers (Macdonald, Hutchinson & Gillet, 1964, 1965; Macdonald, 1968) met two main difficulties. These were the possible selection against segregants of high titre and 'parental genome segregation' in which the haploids of parental type were preferentially recovered. The latter was probably due to the use of parent haploids which differed from each other in chromosomal rearrangements; for example, a reciprocal translocation in one parent would prevent recombination at haploidization between the linkage groups concerned.

The essential aim in this study was to try to overcome the problems that had been met in haploidization. Success in this would permit the building of linkage maps which could provide a basis for a more rational planned programme of breeding. The most promising approach appeared to be through the use of sister strains, differing from each other in only a few mutational steps and, if possible, the use of *p*-fluorophenylalanine (PFA) as an agent to assist in the production of segregant haploids (Lhoas, 1961; Morpurgo, 1961).

METHODS

The media, complete medium (CM) and minimal medium (MM) and methods used were identical to those of Macdonald, Hutchinson & Gillett (1963*a*, *b*, *c*), with the exception that nucleic acid hydrolysates were not added to the CM. The following methods differed from those included in this earlier work.

Haploid and diploid recognition. Heteroallelic diploids were distinguished initially from heterokaryons and parental haploid strains on the basis of spore colour and prototrophy.

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However, the criterion of spore size (Pontecorvo & Sermonti, 1954) is essential for the classification of segregants. In the present work, diploids had a larger mean spore diameter (5·4 μm.) than haploids (4 μm.) and were usually less variable.

Haploidization analysis. A modified version of the PFA technique used with Aspergillus was employed. In order to minimize the possible selection by PFA against certain alleles, colonies were transferred from PFA medium to PFA-free medium. An inhibitory concentration of PFA (0·02 M) was used such that 10% of the diploid spores survived. These conditions were found to be optimal for the recovery of a high frequency of haploid segregants as sectors, provided the smallest colonies were subcultured from PFA after 4 days incubation. While the sectors produced were probably induced by PFA, haploid segregants existing in the treated population could be selected by PFA treatment. However, the latter arose as whole colonies and were avoided in analysis.

Mutants. The symbols used for the u.v.-induced mutants referred to in this work were as follows: *w1*, *w2* (white spore colour); *br1*, *br2* (brown spore colour); *y1*, *y2* (yellow spore colour); *bg1*, *bg2* (bright green spore colour); *cho1*, *cho2* (requirement for choline); *lys1*, *lys2*, *lys3*, *lys4* (requirement for lysine); *an1* (requirement for aneurin); *thio1* (requirement for thiosulphate); *his1* (requirement for histidine); *nic1* (requirement for nicotinamide); *rib1* (inability to grow on ribose as sole carbon source); *aa* (resistance to 8 aza-adenine). Ultraviolet light was chosen as the mutagen since previous studies (Käfer & Chen, 1964; Azevedo & Roper, 1967) had indicated that at 1 to 5% survival translocations did not occur in high frequency in *Aspergillus nidulans*. In expressing the genetic constitution of a strain semi-colons are used to distinguish between genes on different haploidization groups.

RESULTS

The morphologically unstable strain initially selected for study was derived from Q176 by serial irradiation and selection for increased penicillin yield. Like Q176 (Stauffer & Backus, 1954) conidia of this strain when plated onto CM produced colonies of varied growth rate and conidiation due to genetic instability of the strain. About 5% of these colonies had slow growth and poor conidiation, the rest had faster growth rate and denser conidiation. Both classes were relatively stable on further plating but each again produced a proportion (about 5%) of the alternative type. However, three generations of selection for faster growth rate and denser sporulation gave a more stable line. Colonies produced from spore platings of the latter appeared identical; therefore it was used as the parent strain for marker induction and subsequent genetic analysis.

Genetic analysis

Spore colour complementation. Several independently isolated spore-colour mutants were tested in pairs for complementation in heterokaryons and diploids. In the diploid all brown, yellow and bright green mutants complemented with white spore-colour mutants in that the *dark green* wild-type spore colour was produced. Mutants of like colour did not complement. Complementation was also shown by the following combinations in the diploid: *br1*/*y1*, *bg1*/*br1* and *bg2*/*br2*.

No complementation was shown by *br2*/*y1*, *bg1*/*y1* and *bg2*/*y1* and thus it was concluded that spore-colour mutants other than white were mutants of adjacent regions of the genome or at least are related functionally.

Autonomous spore colour was generally observed in heterokaryons with the exception of combinations *y1* with *br1* and *w1* with *br1*. In these cases heterokaryons were dark green.

Linkage. In *Aspergillus nidulans* haploids are derived from diploids as a result of segregation of whole chromosomes largely without crossing over. Coincidence of mitotic crossing over is rare (Pontecorvo & Käfer, 1958). However, in *Verticillium albo-atrum* (Hastie, 1967) and *Aspergillus niger* (Lhoas, 1967) a high frequency of mitotic crossing over makes such coincidence much more probable. Haploidization groups can only be considered as approximations to linkage groups since no meiotic confirmation of inferred linkage is possible. In *Penicillium chrysogenum* the same principles apply. Thus criteria of linkage and non-linkage for any two loci employed in this work have been two- or four-class segregation respectively.

Table 1. *Classification of haploid segregants derived from two diploids after treatment with PFA**

Diploid: *w1 cho1/br1; lys1*		*lys 1*	*lys 1*$^+$	Subtotals	Totals
w1	*cho1*	8	10	18	18
	cho1$^+$	0	0	0	
br1	*cho1*	0	0	0	15
	cho1$^+$	9	6	15	
	Totals	17	16		
Diploid: *y1 cho1/br1; lys1*		*lys 1*	*lys 1*$^+$	Subtotals	Totals
y1	*cho1*	14	6	20	20
	cho1$^+$	0	0	0	
br1	*cho1*	0	0	0	17
	cho1$^+$	3	14	17	
	Totals	17	20		

* Results show linkage of *w1*, *br1*, *y1 and cho1* and independent segregation of *lys1*.

In the former case only the two parental genotypes were recovered in approximately equal frequency, and in the latter case two parental and two recombinant genotypes were recovered, each of the four classes being recovered in approximately the same frequency. Recovery of only one recombinant class in high frequency was considered equivocal for mapping purposes.

The diploids used in defining the existence of at least three haploidization groups in *Penicillium chrysogenum* were as follows: *w1 cho1/br1*; *lys1* (Tables 1 and 2); *y1 cho1/br1*; *lys1* (Tables 1 and 2); *w1 cho1*; *his1/br*; *lys1* (Table 3); *br1*; *his1*; *lys1/w1*; *nic1* (Table 3).

In each case the diploid was dark green, differing slightly in shade from the original green haploid strain from which the components were produced. Thus if normal dark green haploid segregants had been produced they could have been detected. Since none was found, the spore-colour mutants were allocated to the same haploidization group. Linkage to the marker *cho1* (Table 1) substantiates these conclusions.

Table 2 shows the results of analysis of spontaneous haploid segregants from diploids which were later subjected to PFA treatment (see Table 1). A comparison of Tables 1 and 2 clearly shows that the *lys1* allele confers a selective growth advantage to segregants. Only in the presence of PFA is the dominant allele of *lys1* recovered. This is probably achieved because the segregant sectors which arose following PFA treatment came from very poor growing colonies presumed to be aneuploid (see below).

Subsequent analysis has permitted allocation of more than 20 independently isolated mutants to one or other of three haploidization groups (Tables 3 and 4). Included in this is a determinant for unstable morphology '*US*' which was allocated to group III.

The '*US*' determinant was dominant in that the diploid *lys4*; '*US*'/*br1*; *his1*; *nic1* exhibited all the characteristics of the unstable parent. A stable derivative of this unstable diploid was selected. This gave segregants that were morphologically normal. It was concluded that the transition from unstable to stable had been accomplished by either mutation of the '*US*' determinant or mitotic crossing over or non-disjunction to give homozygosity for the stability locus.

Table 2. *Classification of haploid segregants derived from two diploids as sectors, without PFA-treatment*

Diploid: *w1 cho1/br1; lys1*		*lys1*	*lys1*$^+$	Subtotals	Totals
w1	*cho1*	11	1	12	12
	cho1$^+$	0	0	0	
br1	*cho1*	0	0	0	11
	cho1$^+$	11	0	11	
	Totals	22	1		
Diploid: *y1 cho1/br1; lys1*		*lys1*	*lys1*$^+$	Subtotals	Totals
y1	*cho1*	17	0	17	17
	cho1$^+$	0	0	0	
br1	*cho1*	1	0	1	14
	cho1$^+$	12	1	13	
	Totals	30	1		

Table 3. *Classification of haploid segregants from two PFA-treated diploids showing free recombination between three haploidization groups**

Diploid: *w1 cho1; his1/br1; lys1*		*lys1*		*lys1*$^+$		Sub-totals	Totals
		his1	*his1*$^+$	*his1*	*his1*$^+$		
w1	*cho1*	3	4	1	1	9	9
	cho1$^+$	0	0	0	0	0	
br1	*cho1*	0	0	0	0	0	9
	cho1$^+$	4	3	0	2	9	
	Subtotals	7	7	1	3		
	Totals	14		4			
Diploid: *br1; his1; lys1/w1; nic1*		*lys1*		*lys1*$^+$		Sub-totals	Totals
		his1	*his1*$^+$	*his1*	*his1*$^+$		
w1	*nic1*	0	0	1	3	4	10
	nic1$^+$	2	4	0	0	6	
br1	*nic1*	0	0	1	1	2	8
	nic1$^+$	2	4	0	0	6	
	Subtotals	4	8	2	4		
	Totals	12		6			

* *w1* and *br1* are linked, as are *lys1* and *nic1*. Both these groups freely recombine. *his1* freely recombines with both these groups.

Mechanisms of segregation

Haploidization. A slow growing, dark green prototrophic colony derived from PFA treatment of diploid *y1 cho1/br1*; *lys1* was found to produce faster growing normal sectors. The genotypes and ploidy of such sectors were classified at each of three plating generations

of slow-growing prototrophs. In the first generation haploids of genotypes *br1* and *y1 cho1* and diploids of phenotype *y1 cho1* and *y1 cho1*; *lys1* were recovered. In the second generation haploids of genotype *br1*; *lys1* and *y1 cho1*; *lys1* and diploids of phenotype *y1 cho1* were detected. In the final generation similar types to those found for the second generation were recovered. In addition, at each generation dark green prototrophic diploids were found.

Table 4. *Haploidization groups in Penicillium chrysogenum**

Group I
Spore-colour mutants: *w1, w2, y1, y2, br1, br2, bg1, bg2*
Auxotrophs: *cho1, cho2, lys4, an1, thio1*
Resistance: *aa1*

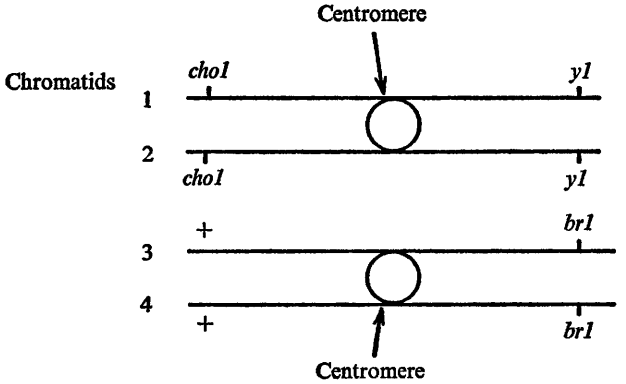
Group II
Auxotrophs: *his1*

Group III
Auxotrophs: *lys1, lys2, lys3, nic1, rib1*
Morphological: '*US*'

* See text for description of allele symbols. Of mutants of like type in any one group, only the spore-colour mutants have been subjected to complementation tests.

It was concluded that the slow-growing prototrophic colony, while being heterozygous for all the known loci, was probably a $2n-1$ aneuploid being monosomic for an unmarked linkage group. This conclusion was reached because the patterns of segregation were very similar to those found by Käfer (1961) when non-disjunction and chromosome loss were postulated as causes of haploidization in *Aspergillus nidulans*.

Mitotic crossing-over. Suspensions of conidia of the dark green (wild-type spore colour) diploid *y1 cho1*/*br1*; *lys1* were treated with a dose of u.v. that gave 50% survival when plated onto CM. A proportion of the resulting colonies were mosaic for conidial colour. Five such mosaics were analysed. Two were a mixture of yellow and brown prototrophic diploids and three a mixture of yellow, brown and dark green diploid prototrophs. Since *y1* and *cho1* are linked (Tables 1 and 4) non-disjunction could not give yellow prototrophs. Therefore it is likely that mitotic crossing over had occurred. Indeed, the yellow and brown prototrophs contained in these mosaic colonies probably represent the reciprocal products of a single mitotic crossing-over event. In addition, it was possible to detect dark green *cho1* homozygotes and therefore it was concluded that *y1* and *cho1* mark different arms of group I. The following diagram illustrates the proposed distribution of markers at the four-strand stage of mitotic division.



A single crossover between the centromere and the spore colour markers gives reciprocal products of segregation with genetic constitution *y1 cho1/y1* (chromatid 1 with 3) and *br1 cho1/br1* (chromatid 2 with 4). Subsequent PFA breakdown of certain of these yellow and brown prototrophs confirmed that they were heterozygous for *cho1*.

From the frequency of yellow prototrophs arising spontaneously as sectors from the centre of normal diploid colonies (1 in 300) it was possible to *estimate* the frequency of mitotic crossing-over for the whole genome (six chromosome arms) at 4% (12/300 × 100). This value is approximately 20 times higher than that found for *Aspergillus nidulans* (Pontecorvo & Käfer, 1958) and approximately five times less than that found in *A. niger* (Lhoas, 1967).

DISCUSSION

This study has shown that problems in undertaking the formal genetic analysis of *Penicillium chrysogenum* through the parasexual cycle can be surmounted. The use of relatively stable sister strains in conjunction with haploidization induced by PFA has enabled two major problems posed in earlier studies to be overcome, namely 'parental genome segregation' and possible selection against alleles.

The way is now open for allocation of a number of loci, which increase penicillin yield, to their haploidization groups. Subsequently, such increases can be combined in a controlled way either by haploidization or mitotic crossing-over to produce desired combinations of alleles.

Recent studies suggest that this has been achieved by haploidization (Ball, 1970). However, it should be possible to recombine genes on the same haploidization group in a controlled way, using mitotic crossing-over by use of heterozygous recessive selective markers such as spore-colour mutants. Yield-increasing loci distal to such markers should become homozygous, thus enabling control to be exerted over the breeding process. An important aspect of this is that more recessive selective markers (e.g. drug resistance) need to be produced.

In addition, the present work has shown that a morphologically unstable determinant, analogous to that described by Stauffer & Backus (1954), has been allocated to a haploidization group. Recent studies with *Aspergillus nidulans* (Bainbridge & Roper, 1966; Azevedo & Roper 1967; Ball, 1967; Nga & Roper, 1967) have provided mechanisms based on partial chromosome duplication that could well explain the unstable system studied here.

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