Hybridization and Selection for Increased Penicillin Titre in Wild-type Isolates of *Aspergillus nidulans*

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SUMMARY

Repeated hybridization and selection among wild-type isolates produced strains of *Aspergillus nidulans* with increased penicillin titre. Four independent selection lines were established, each originating from a sexual cross between two different heterokaryon-incompatible wild-type isolates. In each generation, two selected high-titre sister strains were crossed to produce the next generation. An initial increase in titre was obtained in each line, but after four or five generations of selection the genetic variation was considerably reduced and the rate of response to selection had decreased. From a base population of wild-type isolates with a mean titre of 8.6 units/ml the progeny mean titre was raised to between 16 and 20 units/ml in each line. The gradual nature of the response suggests that a number of genes determine penicillin titre in the wild-type isolates used. The gene action throughout the selection programme was predominantly additive.

INTRODUCTION

The discovery of a parasexual cycle in *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1953) led to forecasts that planned breeding programmes for increased titre and genetic analysis of penicillin production could have a significant influence on strain improvement in industry (Pontecorvo, 1956; Magni, 1961). Subsequent detailed studies of penicillin production in *P. chrysogenum* using parasexual analyses (Sermonti, 1959; Macdonald, Hutchinson & Gillett, 1963*a, b, c*, 1964, 1965) cast doubts on the practical application of hybridization to titre improvement and these reservations do not appear to have changed significantly in recent years (Alikhanian, 1970). Genetic analysis of high-titre industrial strains is impeded by the low frequency of recovery of recombinants among segregants from diploids (Macdonald *et al.* 1965) and by frequent somatic instability (Elander, 1967). Both these difficulties may be attributed to chromosomal aberrations induced by the recurrent mutagenic treatments used in titre improvement (Macdonald, 1968; Käfer, 1969; Azevedo & Roper, 1970). However, Ball (1971) showed that both these problems can be overcome by careful selection of the parental strains.

A number of fungi, in addition to *Penicillium*, produce penicillin. Holt & Macdonald (1968*a, b*) and Macdonald, Holt & Ditchburn (1972) successfully exploited the genetically well-studied species, *Aspergillus nidulans*, to study the genetics of penicillin production; they isolated and mapped single titre-increasing mutations using classical, qualitative genetic techniques. However, except in such circumstances where closely related strains are involved, penicillin titre is likely to be inherited in a quantitative manner (Roper, 1965; Holt & Macdonald, 1968*b*) and the interpretation of these more complex situations is likely to benefit from quantitative analyses (Alikhanian, 1970).

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The techniques of biometrical genetics can be readily applied to haploid meiotic genetic systems (Caten & Jinks, 1975) and have been used to analyse quantitative characters in several species of fungi (Simchen, 1965*a, b*, 1966*a, b*; Papa, Srb & Federer, 1966; Fripp & Caten, 1971). Merrick & Caten (1975*a*) used such quantitative methods to study the extent and genetic control of variation in penicillin titre in wild-type isolates of *A. nidulans*. I continued this investigation, and describe the results of a programme of hybridization and selection for increased titre.

METHODS

General. The isolates of *A. nidulans*, growth media, crossing techniques, induction of spore-colour mutants, penicillin fermentation procedure and assays were all as described by Merrick & Caten (1975*a*).

Heterokaryon compatibility tests. These were carried out using a modified form of the method described by Grindle (1963). Portions (1 ml) of a dense conidial suspension (10^7 to 10^8 conidia/ml) of each of the two strains to be tested were mixed thoroughly and 0.1 ml of the resultant suspension was spread on Plunkett's medium (Plunkett, 1953). The plates were incubated for 6 days at 35 °C and then scored for the presence of mixed conidial heads.

Parental isolates. Two criteria were used in choosing the initial parental isolates from the collection of wild-type isolates available: (i) to maximize the variability available, the parental isolates should be as genetically diverse as possible. This was achieved by ensuring that all the isolates were members of different heterokaryon compatibility (h-c) groups, as isolates from the same h-c group are generally phenotypically and genotypically very similar (Jinks *et al.* 1966; Merrick & Caten, 1975*a*). (ii) The initial parental isolates were chosen from amongst those isolates which produced high titres in the primary screen (Merrick & Caten, 1975*a*). Isolates were therefore selected by starting with the highest-titre isolate and rejecting any isolate which belonged to an h-c group already represented. If the h-c grouping of an isolate was unknown, it was tested for compatibility with the other selected isolates. Eight high-titre isolates (Table 1) were chosen, of which four were already classified into h-c groups and four were unclassified. These will be referred to as the base population.

The selection programme. Since the genetic control of penicillin production in wild-type isolates has been shown to be additive (Merrick & Caten, 1975*a*) a programme of line selection, rather than a more complex scheme, was adopted. This facilitates interpretation of the changes which take place, because the pedigrees of the strains generated are comparatively simple.

The following four crosses were made among the eight selected parental isolates (Table 1): 65×109 , 159×131 , 189×183 , 139×82 . In each cross the second-mentioned parent carried a yellow spore-colour marker. Selection in each line was carried out as follows in all but the first generation. Forty-four single ascospore progeny were obtained at random from a hybrid perithecium and assayed for penicillin production using two fermentation flasks for each progeny strain. The two parent strains from that particular cross and two control strains common to all fermentations were also included in each assay, but with four replicate flasks of each. Each fermentation therefore involved 104 flasks. In the first generation, 50 progeny were assayed and the control strains were not included. The optimum design for experiments of this type has been considered by Merrick & Caten (1975*b*). From each progeny sample, the highest-titre green-spored and the highest-titre yellow-spored strain were chosen as parents of the next generation. This procedure avoided the need for repeated mutagenesis.

A standard analysis of variance, as used by Merrick & Caten (1975*a*), was carried out on

Table 1. *Isolates of A. nidulans used as initial parent strains for the selection programme*

Isolate no.	Heterokaryon-compatibility (h-c) group	Titre* (u/ml)
65	A	11.9
82	—	9.6
109	H	14.4
131	—	11.9
139	E	10.5
159	I	12.7
183	—	11.2
189	—	9.4

—, H-c group unclassified but incompatible with all the other seven selected isolates.

* Penicillin production in primary screen of wild-type isolates of *A. nidulans* (Merrick & Caten, 1975a).

the titres of the progeny from each generation in each selection line and the following statistics were calculated: progeny mean titre; parental mean titre; σ_E^2 , a measure of the environmental variation in the fermentation and the assay; σ_σ^2 , a measure of the variation due to segregation of natural allelic differences affecting penicillin titre; σ_c^2 , a measure of variation associated with the spore-colour marker. The results from each cross were also tested for the presence of non-allelic interaction as described by Merrick & Caten (1975a).

In any selection programme the problem arises of making comparisons between successive generations which have been tested in different experiments on different occasions (Ball, 1973). An attempt was made to overcome this problem by the use of control strains against which other strains could be standardized. The control strains, Birmingham isolates Nos. 65 and 176, were assayed in every experiment during the selection programme with the exception of the first generation crosses. By the end of the programme, data had been collected for these two strains on at least fifteen separate occasions covering a period of fifteen months. While the control strains were in regular use, subcultures were kept on MT agar at 4 °C and renewed from silica gel stocks every six to eight weeks.

RESULTS

Control strain variation

In the course of the selection programme the control strains were assayed on 15 occasions with an overall mean titre of 14.43 ± 0.41 and 5.82 ± 0.33 u/ml for isolates 65 and 176 respectively. The titres observed on any one occasion varied about these means (Fig. 1). Despite this variation the strains maintained their relative titres on all occasions, i.e. on no occasion did the titre of isolate 176 exceed that of isolate 65.

An analysis of variance of the data (Table 2) confirms that the differences in titre from experiment to experiment are significant, although estimation of the 'between-experiments' variance component indicated that this represented only 15 % of the total error variation (both within and between experiments). There was also a significant interaction between the two strains and the different occasions. The batch of corn steep liquor (CSL) used in the fermentation medium was changed between experiments 7 and 8 and the 'between experiments' and 'strains $\times$ experiments' items have been partitioned to determine to what extent this change accounts for the differences between experiments (Table 2). These partitions show that while the CSL batch contributed little to the overall titre variation between

Fig. 1. Variation in penicillin titre of the control strains (○) 65 and (●) 176 of *A. nidulans* during the selection programme. Each point represents the mean titre from four flasks. The batch of CSL used in the fermentation medium was changed between experiments 7 and 8.

Table 2. Analysis of variance for the penicillin titres of the control strains 65 and 176 of *A. nidulans* in fifteen separate experiments

	Degrees of freedom	Mean square	Variance ratio
Between strains	1	2225.6854	311.51*
Between experiments	14		
Between CSL batches	1	32.1902	2.14
Within batches	13	15.0701	2.11†
Strains × experiments	14		
Strains × batches	1	43.0880	6.03†
Strains × experiments within batches	13	9.8453	1.46
Error (between replicate flasks)	87	6.7413	
Pooled error	100	7.1448	

* $P \leq 0.001$, when tested against the pooled error.

† $P = 0.05$ to 0.01 , when tested against the pooled error.

experiments, it was entirely responsible for the interaction between the two strains and the different experiments. Thus the change in CSL resulted in a slight increase in the titre of isolate 65 but a decrease for 176 (Fig. 1).

Despite the minor contribution of the differences between experiments to the total error variation, the information from the control strains was used to standardize the observed progeny mean titres in each generation. Examination of the control strain data showed a positive correlation ($r = 0.45$, $P = 0.05$ to 0.01) between the sum of the control strain titres and the difference between them, suggesting that when the mean of the controls was high the high-titre control showed a greater increase in titre than the low-titre control. Therefore a proportional relationship, with all measurements in u/ml, was used as follows:

$$p = \frac{m}{m_x} \times p_x,$$

where m is the mean of the control strains over all occasions, m_x the observed mean of the control strains on occasion x , p_x the observed mean progeny titre on occasion x , and p is the corrected mean progeny titre. All progeny mean titres quoted have been standardized in this way.

Fig. 2. Response to selection for increased penicillin titre in four independent selection lines established from crosses between wild-type isolates of *A. nidulans*. With the exception of generation P, each point represents the mean titre of 44 progeny each in two replicate flasks in each generation (50 progeny in generation 1). Generation P shows the titre of the two parental wild-type isolates used to establish the line, and each point is based on four replicate flasks from the same experiment as the generation 1 progeny.

The selection experiments

The heritable variation present among the progeny of crosses between wild-type isolates (Merrick & Caten, 1975*a*) permitted a considerable response to selection. Penicillin production was increased by 60 to 160 % in each of the lines after four or five generations of selection (Fig. 2), by which time the response had begun to reach a plateau in two of the lines (2 and 4). Since hybrid perithecia could not be obtained from a cross between the selected strains from the third generation of line 3, selection in this line had to be terminated. The four lines, each of which started from an independent gene pool, behaved in a similar manner and reached titres of between 16 and 20 u/ml from a base population with a mean of 8.6 u/ml. The steady response to selection in each line suggests that a number of genes are involved in the determination of penicillin titre.

Owing to a faulty fermentation, spurious results were obtained for the second generation of line 3, as shown by the abnormally low control strain titres (8.63 and 4.57 u/ml, for strains 65 and 176, respectively). Since the progeny mean titre could not be corrected against the control strains because many of the progeny had a zero titre, the progeny mean for this generation was calculated from a small repeat experiment in which the two parental strains were assayed; the calculation was based on the assumption that the gene action is additive (Merrick & Caten, 1975*a*) so that progeny mean equals parental mean.

Changes in the amount of genetic variation for penicillin titre in the progeny also reflect the progress of selection. Because the rate of inbreeding was very high (the system is one of sib-mating with selection) the amount of genetic variation, σ_a^2 , was expected to decrease

Fig. 3. Changes in the components of variation for each generation with selection for increased penicillin titre in four selection lines of *A. nidulans*. Each generation in each line comprised 44 progeny (50 in generation 1). ●, $\hat{\sigma}_G^2$ and ○, $\hat{\sigma}_E^2$ are estimates of the heritable and environmental components of variation, respectively.

rapidly. Even in the absence of selection, σ_G^2 should theoretically be halved in each generation. This trend was particularly clear in lines 1 and 4 where $\hat{\sigma}_G^2$ (the estimated value of σ_G^2) was reduced to zero in four or five generations (Fig. 3).

In line 2, the initial amount of genetic variation was extremely small and showed only a slight increase before dropping to zero in the fourth generation. This initial low level suggests that the parental isolates were almost isogenic for penicillin genes. The rise in $\hat{\sigma}_G^2$ in the fifth generation of this line may be attributable to a rare recombination event leading to the break-up of a balanced polygenic combination. Events of this kind were observed by Papa *et al.* (1966) when selecting for increased growth rate in *Neurospora crassa*. The estimate of σ_G^2 in the second generation of line 3 was spuriously inflated by a number of progeny which gave a zero titre as a result of the faulty fermentation.

The estimate of σ_E^2 differed considerably from one occasion to the next (Fig. 3), reflecting the sensitivity of penicillin titre to environmental variables. There is, however, no evident trend in $\hat{\sigma}_E^2$ (the estimated value for σ_E^2) as might have been expected if the strains were changing in stability with selection.

Tests for non-allelic interaction were significant in only three of the 17 crosses and of these three only one was highly significant ($P < 0.001$). The results are therefore consistent with a model of additive gene action as found in crosses between wild-type isolates (Merrick & Caten, 1975a).

Effects of conidial-colour markers on titre

Preliminary studies of 13 white and 17 yellow spore-colour mutants, all isolated independently in three wild-type isolates, indicated that neither white nor yellow mutations had a significant effect on titre (Merrick, unpublished). However, in four out of seven crosses between wild-type isolates (Merrick & Caten, 1975a), yellow-spored progeny had a signifi-

cantly lower titre than green-spored progeny; a similar effect was found in 10 of the 16 crosses in this selection programme. The mean of the yellow-spored progeny was never significantly greater than the mean of the green-spored progeny.

This observed association between the *y* allele and reduced titre could arise as a result of linkage between *y* and alleles for low titre, or as a result of a pleiotropic effect of the yellow mutation. The possibility that the observed effect was due to linkage was examined in two crosses between Birmingham isolates 183 and 189 in which a yellow spore-colour marker was used alternately in each parent. In both crosses the yellow-spored progeny had a significantly lower titre than the green-spored progeny, indicating pleiotropy rather than linkage. The nature of this pleiotropic effect is not understood.

DISCUSSION

Hybridization and strain improvement. This work demonstrates that it is possible to obtain significant improvement in penicillin titre by a programme of hybridization and selection. This response to hybridization and selection confirms the existence of considerable potential variation for penicillin production among wild-type isolates of *A. nidulans* and demonstrates the importance of recombination for the attainment of maximum phenotypic expression. Although only naturally-occurring allelic differences have been involved in this work, there is no reason to suppose that mutagenically-induced variation could not be exploited in a similar manner.

The success of this programme indicates that hybridization can be a useful tool for the production of improved micro-organisms and that quantitative genetic analysis can provide valuable information about polygenic systems involved in antibiotic production. There is considerable scope for the application of such methods to the genetic analysis of industrially important micro-organisms. Adaptation of the methods described here would be necessary where non-meiotic systems were involved, but these difficulties should be surmountable by the use of appropriate selective techniques (Caten & Jinks, 1975).

Selection experiments in fungi. Systematic selection experiments in fungi have been carried out using a number of species. Two studies on linear growth rate, one in *Schizophyllum commune* (Simchen, 1966*a*) and the other in *N. crassa* (Papa *et al.* 1966), are directly comparable with this present work. There was a rapid drop in the genetic variation and an associated decrease in the rate of response to selection in the experiments of both Papa and Simchen which were very similar to those described here for penicillin titre. Two factors might be expected to have contributed to this pattern: (i) the programme of sib-mating used in all three studies should alone lead to a halving of the genetic variance in each generation, ignoring any effects of selection; (ii) fixation of advantageous alleles through selection should occur more rapidly in haploids than in diploids, where dominance in the direction of selection will result in heterozygotes as well as homozygotes being chosen. Thus line selection with its associated high levels of inbreeding has led to significant changes in the characters concerned, but both theoretical considerations and observed results suggest that where the aim is simply to achieve the maximum response, any one line should not be continued for more than five or six generations. Frequent introduction of new genetic variation from other strains or selection lines is necessary to replenish the gene pool, and the optimum selection strategy should therefore be designed to accommodate this from the outset.

The increase in titre achieved in each selection line in the present study may be due to selection of the same or of different genes for increased titre in each line. To distinguish

between these possibilities, crosses can be made between the lines. An analysis of the selected strains along these lines, is discussed in the following paper.

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