Selection for Increased Penicillin Titre Following Hybridization of Divergent Lines of *Aspergillus nidulans*

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Sexual hybridization of two divergent lines of *Aspergillus nidulans*, which had been selected for increased penicillin titre through successive cycles of mutagenesis, released considerable variation for this character. The recovery of segregants with titres equivalent to that of the unselected ancestor suggested that mutations in different genes had been selected in the two lines. However, complementary segregants with substantially improved titres were not found, indicating interactions, probably of a duplicate nature, among the induced mutations. All the genetic variation released by hybridization was fixed following two generations of selection for high titre, but only a small gain over the initial selection lines was achieved. Hybridization of divergent strains has been widely advocated as a means of strain development. The failure to achieve the anticipated gains in this programme is attributed primarily to the unfavourable interactions amongst the induced mutations. Whether similar interactions occur generally in crosses between strains selected by mutagenesis remains to be established and will be an important factor in determining the contribution of recombinational approaches to yield improvement.

INTRODUCTION

There has been considerable discussion of the use of recombination for strain improvement in antibiotic-producing micro-organisms (for example, Calam, 1964; Sermonti, 1969; Alikhanian, 1970; Elander & Espenshade, 1976), and systems of recombination have been developed in several industrially important species (for review, see Hopwood & Merrick, 1977). Particularly attractive is the possibility of combining yield-enhancing alleles from divergent sources into one strain through hybridization followed by selection amongst the progeny. However, extensive attempts to breed improved strains of *Penicillium chrysogenum* through parasexual hybridization were largely unsuccessful (Sermonti, 1959; Macdonald *et al.*, 1965; Macdonald, 1966; Elander *et al.*, 1973) and it is generally considered that recombination has to date contributed little to practical strain improvement (Elander *et al.*, 1977). Possible reasons for the failure of these initial attempts at breeding have since been identified (Ball, 1971, 1973), and currently there is a renewed interest in recombination as a means of strain improvement (Hopwood, 1977; Hopwood & Chater, 1980; Ball, 1978). The practical value of hybridization has been recently demonstrated by the isolation from a cross between two divergent lines of *Cephalosporium acremonium* of a segregant showing a 40% increase in cephalosporin C titre, together with other improvements (Hamlyn & Ball, 1979).

Soller (1980) developed models for the response of microbial populations to selection programmes involving mutagenesis alone or mutagenesis in combination with hybridization. Comparisons of the response to selection predicted by these two models indicated that under certain conditions the gains in the combined programme may exceed those in the mutagenesis

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programme and therefore that it may pay to divide the mutation stages into two divergent lines which can subsequently be crossed. The extent to which the conditions favouring hybridization will be realized in practice can only be determined by experiment. This paper describes hybridization and selection experiments which are very similar to the model programmes considered by Soller (1980). The results provide tests for many of the critical conditions of the models and permit some evaluation of the benefits of recombination in yield-improvement programmes.

Two divergent strains of *Aspergillus nidulans*, A6-9 and B6-27, were independently selected from isogenic ancestral strains by recurrent mutagenesis (Simpson & Caten, 1979*b*). Selection was for increased penicillin titre in shake-flask fermentations and each strain showed around a threefold increase in productivity over its unselected ancestor. Although *A. nidulans* is not a commercial antibiotic producer it has proved valuable as a model organism for genetic studies of penicillin production (Macdonald & Holt, 1976), and the derivation of A6-9 and B6-27 was closely comparable to the procedure used industrially for yield improvement in antibiotic-producing organisms (Davies, 1964; Demain, 1973; Elander & Espenshade, 1976). Information obtained from studies of A6-9 and B6-27 should therefore be directly relevant to the genetics of antibiotic yield in improved industrial strains. Backcrosses of the two selected strains to the ancestral genotype revealed that the increased yield of each was due to a polygenic system involving both additive and non-additive gene action (Simpson & Caten, 1980). A diploid constructed between A6-9 and B6-27 gave a low titre implying that the titre-increasing mutations ('+' mutations) in the two lines were non-allelic (Simpson & Caten, 1980). We here report an attempt to select recombinants with further increased penicillin titre from a cross between A6-9 and B6-27. Following the initial cross, two generations of selection and hybridization were initiated from the progeny population. Sexual rather than parasexual hybridization was used because (1) it avoids the need to select haploid segregants and therefore facilitates taking a random sample of progeny (Caten & Jinks, 1976), (2) the theory and methods of quantitative genetics can be readily applied (Caten, 1979) and (3) the rate of intrachromosomal recombination is much higher.

METHODS

Strains. The origins of the ancestral strains of *Aspergillus nidulans*, strain A (ATCC 42369) and strain B (ATCC 42370), have been described by Simpson & Caten (1979*a*) who referred to them as 7/142 and 7/151, respectively. Strain A carries the marker *biA1* and strain B carries the markers *pabaA1*; *wA3*. These markers do not significantly affect penicillin titre under the fermentation conditions used (Simpson & Caten, 1980). Strains A and B are both descended from the 'Glasgow' wild-type, NRRL 194. Both carry the standard chromosome complement and are isogenic for genes affecting penicillin titre (Simpson & Caten, 1980). The improved strains A6-9 (*biA1*; $\Sigma penA$) and B6-27 (*pabaA1*; *wA3*; $\Sigma penB$) were derived from A and B, respectively, through six cycles of mutation and selection for increased penicillin titre (Simpson & Caten, 1979*b*). Averaged over at least six separate fermentations these strains gave the following mean penicillin titres (range in parentheses): A = 5.7 u ml^{-1} (3.0–7.4); A6-9 = 14.2 u ml^{-1} (11.2–20.5); B = 5.9 u ml^{-1} (3.2–7.8); B6-27 = 15.5 u ml^{-1} (11.0–19.6). $\Sigma penA$ and $\Sigma penB$ denote the '+' mutations accumulated in A6-9 and B6-27, respectively. Other gene symbols are standard for *A. nidulans* (Clutterbuck, 1973).

General. Media, methods of culture, fermentation and bioassay procedures and statistical analyses were all as described previously (Simpson & Caten, 1979*a, b*). Crosses were carried out by the sexual cycle using single perithecial analysis as described by Merrick & Caten (1975).

RESULTS

Hybridization of A6-9 and B6-27

Strains A6-9 and B6-27 were crossed twice and a sample of progeny taken on each occasion. The first sample, designated S0, formed the base population for a subsequent programme of hybridization and selection and contained 95 randomly selected progeny. The second sample, designated SX, was chosen to test for any association between the induced

Fig. 1. Distribution of penicillin titres among progeny from crosses $A \times B$ (a), $A6-9 \times B6-27$, S0 (b) and $A6-9 \times B6-27$, SX (c). The titres of A, B, A6-9 and B6-27 are shown by arrows. The bars indicate the progeny mean titres.

titre mutations and the markers (*biA*, *pabaA*, *wA*) and consisted of six progeny of each of the eight marker genotypes arising from the cross. The titres of the S0 progeny were determined in two separate fermentation experiments. In both experiments the 95 progeny were each fermented in one flask. These flasks were sampled and assayed following the procedure previously used for populations of around 100 strains (Simpson & Caten, 1979*b*). The SX progeny were each fermented in two replicate flasks in a single experiment and were assayed following the general procedure for populations of around 50 strains (Simpson & Caten, 1979*a*). Two replicate flasks of A, B, A6-9 and B6-27 were included in all three experiments.

The distributions of the progeny titres in samples S0 (mean of the two experiments) and SX are shown in Fig. 1(b) and (c), respectively. Results previously obtained for a sample of progeny from the cross between the ancestral strains (Simpson & Caten, 1980) are included as a control (Fig. 1*a*). Strains A and B performed similarly in all experiments but A6-9 and B6-27 gave higher titres in the SX fermentation than in the two S0 fermentations. In the absence of a consistent effect upon the performance of these four standard strains no attempt was made to correct the progeny titres for differences in the fermentation conditions between experiments. All three progeny populations showed continuous and unimodal distributions (Fig. 1). The means of the two $A6-9 \times B6-27$ progenies were higher than that for the control population, confirming the genetic basis of the high titres of the selected strains. Furthermore, the range of titres amongst the progeny of $A6-9 \times B6-27$ was markedly greater than that amongst the control population, reflecting the segregation and recombination of alleles affecting penicillin production. In both $A6-9 \times B6-27$ crosses a few progeny were recovered with titres in excess of both parental isolates; however, the majority were less productive than their parents and a few produced no more penicillin than the unselected ancestors A and B (Fig. 1*b, c*).

Analyses of variance for the data from the three progeny samples are summarized in Table 1. The variation within each progeny sample was partitioned into that between and that

Table 1. *Summary of analyses of variation in penicillin titre ($\log u\ ml^{-1}$) among progenies from $A \times B$ and $A6-9 \times B6-27$*

Cross	(a) Tests of significance									
	Between progeny		Between marker genotypes		Within marker genotypes		Between blocks		Progeny $\times$ blocks	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
$A \times B^\dagger$	47	0.010	7	0.016	40	0.009	1	0.149*	47	0.012
$A6-9 \times B6-27, S0$	94	0.044*	7	0.065	87	0.043*	1	0.691*	94	0.017
$A6-9 \times B6-27, SX$	47	0.042*	7	0.031	40	0.044*	1	0.001	47	0.012

(b) Components of variance

Cross	Genetic (σ_G^2)	Blocks (σ_B^2)	Environmental (σ_E^2)
$A \times B^\dagger$	0.000	0.003‡	0.012
$A6-9 \times B6-27, S0$	0.014‡	0.007‡	0.017
$A6-9 \times B6-27, SX$	0.015‡	0.000	0.012

d.f., degrees of freedom; M.S., mean square.

† Data from Simpson & Caten (1980).

* $P < 0.001$.

‡ Significantly different from zero.

within marker genotypes. In no case was the between marker genotypes mean square (M.S.) significantly greater than the within marker genotypes M.S. This lack of any effect of the three markers on penicillin titre is consistent with the backcrosses of A6-9 and B6-27 (Simpson & Caten, 1980) and means that the between and within marker genotype M.S. values can be pooled to give a between progeny M.S. This between progeny M.S. was highly significant for the two progenies from A6-9 $\times$ B6-27 but not for $A \times B$ (Table 1a). S0 and SX gave similar estimates for the amount of genetic variation (σ_G^2) released in A6-9 $\times$ B6-27 which clearly contrast with the zero estimate for $A \times B$ (Table 1b). The higher estimates of the environmental component (σ_E^2) and of the variance between blocks (σ_B^2) for S0 (Table 1b) may be attributed to the fact that the replicates in this case were fermented in different experiments. These analyses confirm the segregation of alleles affecting penicillin titre in the cross between the divergent selection lines. Since the cross between the ancestral strains does not segregate, these genetic differences between A6-9 and B6-27 must have been induced during the mutagenesis programmes.

The progeny of A6-9 $\times$ B6-27 were not symmetrically distributed about the parental mean as expected with solely additive gene action (Caten & Jinks, 1976). In both samples there was an excess of progeny with titres below the mid-parental value and this was particularly marked for SX (Fig. 1b, c). The deviation of the progeny mean from the mid-parental value gives an estimate of the contribution of directional non-additive gene effects [i] to the parental phenotypes (Mather & Jinks, 1971; Caten, 1979). Whether these estimates are significantly different from zero can be tested with a 't' test. Non-additive gene effects (epistasis) were not apparent in $A \times B$ (Table 2a). (This was expected since the overall analysis indicated no genetic variation in this cross.) In both samples of progeny from A6-9 $\times$ B6-27, however, the progeny mean was less than the mid-parental value, giving positive estimates of [i] (Table 2a). The consistency of this effect over the two samples and its statistical significance for SX imply that the '+' mutations in A6-9 and B6-27 are partially non-additive in their effects.

Recurrent hybridization and selection

Following the initial cross (S0) between A6-9 and B6-27, two generations of hybridization were carried out using, in each case, high-titre progeny selected from the previous generation as parents. The highest green-spored and the highest white-spored segregant in S0 were crossed to produce the first selection generation (S1). Forty-five randomly selected S1

Table 2. Tests for epistasis in crosses $A \times B$, $A6-9 \times B6-27$ and in two subsequent generations of hybridization and selection

Cross	P ₁ *		P ₂ *		Parental mean titre (u ml ⁻¹)	Progeny mean titre, <i>F</i> ₁ (u ml ⁻¹)	<i>i</i> †
	Strain	Titre (u ml ⁻¹)	Strain	Titre (u ml ⁻¹)			
(a) Initial hybridization							
A × B	A	6.3	B	5.8	6.1	6.0	+0.1
A6.9 × B6-27, S0	A6.9	11.2	B6-27	14.4	12.8	11.8	+1.0
A6.9 × B6-27, SX	A6.9	18.5	B6-27	19.6	19.1	15.2	+3.9‡
(b) Selection generations							
S1	S0-88	13.9	S0-87	15.1	14.5	15.2	-0.7
S2	S1-33	16.1	S1-32	16.3	16.2	17.0	-0.8

* P_1 , green-spored parent; P_2 , white-spored parent.

† $|i| = \frac{1}{2}(\bar{P}_1 + \bar{P}_2) - \bar{F}_1$.

‡ Significantly different from zero: test of significance carried out on log u ml⁻¹.

Fig. 2. Response to selection for increased penicillin production following hybridization of A6-9 and B6-27. (a) Changes in the progeny mean titre (○) and in the mean of the highest green- and white-spored segregants, i.e. the strains selected as parents for the next generation (●). (b) Changes in the genetic (○) and environmental (●) components of variance.

progeny were each assayed in two replicate shake flasks following the procedure used for SX. On the basis of these results, the highest green-spored and white-spored segregants in S1 were selected and crossed to give the second selection generation (S2). Forty-six S2 progeny were assayed as for the S1 progeny. Selection of parents for the S1 and S2 generations was based on titre and conidial colour without regard to auxotrophic markers. As a result *bia1* was lost following the S0 generation, but *paba1* continued to segregate in the S1 and S2 generations. Although the requirement for the parents of S1 and S2 to be of different conidial colour prevented selection of the two absolute highest-yielding segregants it avoided the need to reintroduce markers for the identification of hybrid perithecia at each generation. Two replicate flasks of A, B and the appropriate parents were assayed together with the S1 and S2 progenies. The titres of A and B in S0, S1 and S2 were similar and no attempt was made to correct the progeny titres for differences in the fermentation conditions between generations.

The progeny mean titre increased by almost 45% from 11.8 u ml⁻¹ to 17.0 u ml⁻¹ between S0 and S2 (Fig. 2a). This was accompanied by a fall in the amount of genetic variation in the progenies (Fig. 2b). Some genetic variation remained after one generation of selection (S1) but none could be detected after two (S2) (Table 3). As in the S0 generation, the markers (*paba1* and *wal* only) had no effect on penicillin titre in the S1 and S2 generations (Table 3) and the total between strains M.S. has been used to estimate σ_b^2 . Thus all the genetic variation

Table 3. *Summary of analyses of variation in penicillin titre ($\log u\text{ ml}^{-1}$) among progenies from two successive generations of hybridization and selection*

Cross	Between progeny		Between marker genotypes		Within marker genotypes		Between blocks		Progeny $\times$ blocks	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
S1	44	0.012*	3	0.002	41	0.012*	1	0.028*	44	0.004
S2	45	0.004	3	0.001	42	0.004	1	0.000	45	0.004

d.f., degrees of freedom; M.S., mean square. * $P < 0.001$.

released by hybridization of the divergent strains had been fixed after two generations; consequently no further response was expected and the programme of recurrent hybridization and selection was terminated. The change from replication of fermentations over experiments (S0) to replication within an experiment (S1 and S2) led to a drop in the environmental component of variation (Fig. 2*b*).

The parental mean titres also increased during selection, in this case from 12.8 u ml^{-1} in S0 (mean of A6-9 and B6-27) to 16.2 u ml^{-1} in S2. However, this increase is more apparent than real since the titres of A6-9 and B6-27 in S0 (Table 2*a*) were low relative to their average performance (14.2 u ml^{-1} and 15.5 u ml^{-1} , respectively). The highest yields obtained among the S2 progeny (18 u ml^{-1}) were higher than the average values for A6-9 and B6-27 but not greater than the maximum titres recorded for these strains. Thus, although the recombination programme gave some improvement over the initial mutation-selected strains, the gain was small, i.e. around 20% of the mean of A6-9 and B6-27. Estimation of $[i]$ for the S1 and S2 generations gave non-significant negative values (Table 2*b*) indicating that the genes responsible for the non-additive effects in the S0 generation had been fixed after one generation of selection.

DISCUSSION

Hybridization of A6-9 and B6-27 released considerable genetic variation for penicillin titre indicating that many of the '+' mutations in the two lines involved different genes. In fact the recovery of some progeny with titres equivalent to the ancestral strains A and B (Fig. 1*b, c*) suggests no overlap between the two sets of mutations. This confirms the conclusion from a complementation test that A6-9 and B6-27 carry non-allelic mutations (Simpson & Caten, 1980). The involvement of different genes in increasing penicillin titre in independently selected strains of *A. nidulans* has also been found with major mutations (Ditchburn *et al.*, 1976) and with natural variation (Merrick, 1975*b*). Deductions concerning the extent of allelism between divergent strains of *P. chrysogenum* from the results of parasexual crosses are complicated firstly by variation in yield between independent diploids from the same original parents (Macdonald *et al.*, 1965), and secondly by the parental genome segregation observed in these crosses (Macdonald *et al.*, 1964, 1965).

The cross between A6-9 and B6-27 showed strong evidence of non-additive gene action. Some non-additivity of the mutations in these two strains had previously been suggested by the fact that the observed titre of diploid A6-9/B6-27 was lower than that predicted by a model involving additive and dominance effects only (Simpson & Caten, 1980). Furthermore, backcrosses of A6-9 and B6-27 to the ancestral genotype indicated duplicate interactions within each strain (Simpson & Caten, 1980). Duplicate epistasis would also account for the A6-9 $\times$ B6-27 progeny distribution, with the difference in the sign of $[i]$ between the two types of cross ($[i]$ was negative in the backcrosses but positive for A6-9 $\times$ B6-27) resulting from the fact that the parents for the backcrosses were in coupling whilst those for A6-9 $\times$ B6-27 were in repulsion. (The values of $[i]$ for the backcrosses given by Simpson & Caten, 1980, are in $\log u\text{ ml}^{-1}$.) The results for the backcrosses and A6-9 $\times$ B6-27 therefore suggest that on

average the individual '+' mutations selected during the mutagenesis programme exert their biggest effects on a low-titre background and that accumulation of mutations through mutagenesis or recombination produces progressively decreasing gains. However, this epistatic component cannot account for all the genetic variation since segregants with higher titres than the parents were recovered from A6-9 $\times$ B6-27, indicating an additive component. Indeed, some additivity is essential for the selection of successive mutations in a recurrent mutagenesis programme. Estimation of the relative contribution of these additive and non-additive effects to the genetic variation would require raising further generations (Mather & Jinks, 1971).

Three major mutations increasing penicillin yield in *A. nidulans* also interacted in a duplicate manner such that recombinant strains carrying pairwise combinations of these mutations produced no more penicillin than strains carrying the higher-yielding single mutation on its own (Ditchburn *et al.*, 1976). In addition, results similar to those for A6-9 $\times$ B6-27 were obtained from a cross between two divergent lines of *Cephalosporium acremonium*, with many progeny producing less cephalosporin C than the lower-yielding parent but few producing more than the higher-yielding parent (Hamlyn & Ball, 1979). However, one segregant among 600 tested gave a 40% yield improvement over the higher-yielding parent, demonstrating that valuable gains could still be achieved through recombination. Not all induced mutations increasing antibiotic yield show such undesirable interactions. Ball (1973) reported that parasexual segregants of *P. chrysogenum* carrying two independently induced mutations (t_2 and t_3) gave penicillin titres in excess of any recorded previously. Hence the effects of t_2 and t_3 appear additive or even complementary.

Two generations of selection from the progeny of A6-9 $\times$ B6-27 gave only a small further increase in productivity. Small realized gains following hybridization of divergent lines may result from several factors including: (1) overlap of mutations, (2) low heritabilities, (3) failure of recombination, (4) epistasis and (5) genetic drift (see Soller, 1980, for elaboration of 1, 4 and 5). Let us consider the possible impact of each of these factors on this recombination and selection programme. It has been concluded above that the '+' mutations in A6-9 and B6-27 involve different genes. Estimates of heritability (Merrick & Caten, 1975) for the A6-9 $\times$ B6-27 crosses (S0, $h^2 = 0.45$; SX, $h^2 = 0.55$) are similar to those for populations produced by crossing wild-type isolates (Merrick & Caten, 1975; Merrick, 1975*a*), by crossing selected lines (Merrick, 1975*b*) and by mutagen treatment (Simpson & Caten, 1979*a*); selection was highly effective from these latter populations. Failure of recombination may arise through inefficiency of the genetic system employed or linkage. Meiotic recombination in *A. nidulans* is highly efficient (Cove, 1977) and the markers in A6-9 $\times$ B6-27 recombined as expected. Furthermore, the recovery of low-titre segregants (Fig. 1*b, c*) in small progeny samples indicates that the titre alleles are not linked. Factors 1, 2 and 3 did not therefore apply. As regards factor 4, however, there is strong evidence that the '+' mutations interact in a duplicate manner. These interactions would markedly reduce the yield advance achieved by selecting segregants carrying all or most '+' mutations relative to that expected with an additive system. Since only two parents were selected in each generation it is likely that some favourable alleles were lost by chance (factor 5). These considerations suggest that the small magnitude of the gains from hybridization of A6-9 and B6-27 was due primarily to epistasis, with genetic drift as a possible secondary factor. This conclusion is supported by comparisons with a selection programme carried out using natural genetic variation for penicillin production in *A. nidulans* (Merrick, 1975*a, b*). In this case hybridization of independently selected lines allowed a further substantial increase in titre. The methods used by Merrick were virtually identical to those used here and both his and the present inter-line cross indicated little overlap of mutations and similar heritabilities. The major difference was in the type of gene action which was predominantly additive for the natural variation. This contrast between the genetic architecture of induced and natural polygenic systems suggests that the latter is a selected sample of the allelic differences that can be generated by mutagenesis.

The selection of A6-9 and B6-27 (Simpson & Caten, 1979*b*) and the subsequent selection amongst their progeny described here may be considered as a single strain development programme with a phase of recurrent mutagenesis and a phase of recurrent recombination, each consisting of several cycles. The largest gains per cycle were realized during the mutagenesis phase and, as a means of generating the most productive strain with the resources available, the division of the mutagenesis phase into two parallel lines and the subsequent hybridization of these lines was not effective. For the same total effort a single mutation line could have been carried through 15 cycles and would have undoubtedly shown substantially greater improvement. This contrasts with the conclusion of Soller (1980) that where the genetic variation due to mutagenesis (σ_M^2) is large relative to the measurement errors (σ_E^2) (i.e. $\sigma_M^2 \geq \sigma_E^2$) and the degree of overlap of the mutations is small, both conditions which apply in the present case, a combined programme of mutagenesis and recombination should show a greater gain than a programme based on mutagenesis alone. However, Soller (1980) did recognize that the advantage of the combined programme would be less in the presence of epistasis and in the absence of a deleterious effect of mutagenesis on the population mean. Mutagenesis did depress the mean titre of the *A. nidulans* populations but this reduction was small and probably due to the average greater effect of titre-decreasing mutations rather than to their higher frequency (Simpson & Caten, 1979*a*).

These genetic analyses of A6-9 and B6-27 point to the development under recurrent mutagenesis of complex polygenic systems and suggest that unfavourable gene interactions may limit the gains that can be achieved through recombination of divergent lines. Whether similar genetic control would evolve with other organisms and products, or indeed with different mutagenic treatments, can only be determined by experiment. Further quantitative genetic studies of antibiotic yield are required to establish general values for the relevant parameters and to aid choice of the best combination of mutation and recombination in strain development.

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