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Recurrent Mutation and Selection for Increased Penicillin Titre in *Aspergillus nidulans*

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Recurrent mutation and selection has been used to increase penicillin titre in two closely related strains of *Aspergillus nidulans*. A selection programme was initiated from each of the two strains (programmes A and B) and continued through six cycles of mutation and selection. Near-ultraviolet light in conjuction with 8-methoxypsoralen was employed as the sole mutagen throughout programme A and ethyl methanesulphonate as the sole mutagen throughout programme B. Excluding the first cycle of A, where only 50 strains were assayed, the selection programmes were identical. In each programme, 100 survivors were assayed for penicillin titre after each mutagenic treatment and, on the basis of a single yield test, the best five strains were picked and carried forward to the next cycle. In both selection programmes, a near 300 % increase in penicillin titre was achieved. This yield advance illustrates the effectiveness for strain development of experimental designs involving successive cycles of mutagenesis with a single-stage screen and the selection of the top few percent survivors in each cycle.

INTRODUCTION

Major increases in the production of secondary metabolites have been achieved in strain development programmes through the accumulation of minor mutations in successive cycles of mutagenesis, empirical screening and selection (Sermonti, 1969; Elander & Espenshade, 1976). This stepwise improvement suggests that the potency of the resultant strains would be determined by a polygenic system with the individual factors interacting in a non-additive manner. This expectation could be tested by analyses of improved strains using the techniques of quantitative genetics (Caten & Jinks, 1976). The present paper describes the derivation through a programme of recurrent mutation and selection of the strains required for this test. *Aspergillus nidulans* was chosen as the test organism since the genetic system of this species has been highly developed (Clutterbuck, 1974) and at least certain strains produce an antibiotic which is indistinguishable from penicillin (Holt & Macdonald, 1968).

Preliminary experiments designed to facilitate the choice of mutagen and mutation conditions for this strain development programme have been described previously (Simpson & Caten, 1979). Three mutagens – ethyl methanesulphonate (EMS), near-ultraviolet light in the presence of 8-methoxypsoralen (8MOP) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) – were used in these preliminary experiments. Of these, NTG was the most active in increasing the variability for penicillin titre and in producing titre-increasing variants. However, because of the difficulties experienced in attaining reproducible survival levels and because of safety considerations (Bridges, 1976), it was decided not to use NTG. The remaining two mutagens (EMS and 8MOP) were therefore chosen for two independent

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selection programmes. This combination of mutagens has the added advantage that in *Escherichia coli* at least they act through different mutational pathways, 8MOP being dependent upon a functional *exrA*$^+$/*recA*$^+$ system while EMS is not (Bridges, 1976). Studies of the dose–response relationship for the production of titre-increasing mutants by 8MOP and EMS suggested that mutant frequency was largely independent of dose over the range giving 28 to 4% survival but declined at survivals of less than 1% (Simpson & Caten, 1979). With both mutagens, treatments giving survival levels around 10% produced titre-increasing mutants at a frequency not significantly different from the maximum observed with that mutagen, and since it was desirable for comparative purposes to use the same survival level for the two mutagens (Munson & Goodhead, 1977), these were adopted as the standard treatments throughout. Mutants with increased potency frequently show changes in colony morphology and the pre-selection of morphological mutants for yield testing has been suggested (Demain, 1973). However, examination of the relationship between changes in penicillin titre and colony morphology for the strains of *A. nidulans* indicated that there was no advantage to be gained from such pre-selection (Simpson & Caten, 1979). Accordingly, both mutagens were used at doses and under conditions previously shown to give 10% survival and strains were sampled at random from the survivors for yield testing.

The design of a programme of mutation and selection involves many considerations of which three of the most important are: (1) the size of the population screened (N_1); (2) the number of strains carried forward to the next cycle of mutagenesis (N_2); (3) the number of screening stages used to reduce N_1 to N_2. The ratio N_2/N_1 indicates the selection intensity. Since titre-increasing mutants occur at low frequency, N_1 is normally a compromise between the desirability to screen a large population on the one hand and the limitations posed by the facilities available on the other. In the present programme, available manpower and shaker space set an upper limit of 100 on the number of shake-flask fermentations that could be run on one occasion. This capacity could be most efficiently used by testing 100 different strains, each in a single flask (Y. Zograf, quoted in Alikhanian, 1962); therefore N_1 was set at 100. Although small, samples of this size should include one or more titre-increasing mutants since the mutagenic treatments used produce such mutants at frequencies of 5% or more (Simpson & Caten, 1979). Regarding (2) and (3), the usual procedure in strain development programmes has been to carry forward to the next cycle of mutagenesis only the single highest-yielding strain from the population screened (i.e. $N_2 = 1$) (Backus & Stauffer, 1955). Because of the fermentation and assay errors involved, reliable selection of the most potent mutant necessitates the adoption of multi-stage screens in which the number of strains is progressively decreased and the level of replication increased (Backus & Stauffer, 1955; Alikhanian, 1962; Calam, 1964). Selecting a single strain in this way therefore entails a major investment of time and labour in identifying the best strain. This would no longer be necessary if several strains were carried forward, thereby increasing the probability of selecting a significantly improved strain. The smaller increase in titre expected in any one cycle as a result of the reduced selection intensity should be more than offset by the increased number of cycles that could be accomplished in a fixed time period.

Davies (1964) used computer simulations to explore the effects of different distributions of mutants, selection intensities, number of screening stages and testing standard errors on the rate of advance in antibiotic yield. His results suggested that under normal conditions selection intensities of 2·5% achieved through a two-stage screen should be most efficient. Under favourable conditions with a high frequency of desirable mutations and low testing errors, a single-stage screen coupled with a decreased selection intensity could be adopted without decline in the rate of advance (Davies, 1964). Since the frequency of titre-increasing mutants in the present system was high (>5%; Simpson & Caten, 1979) and the testing errors low (coefficient of variation 12·6%), it was decided to use a single-stage screen together with a selection intensity of 5%, i.e. $N_2 = 5$.

METHODS

General. The two initial strains of *Aspergillus nidulans*, 7/142 (*biA1*) and 7/151 (*pabaA1*; *wA3*), were isogenic except for the genetic markers and were translocation-free (Simpson & Caten, 1979). The media for routine growth, conidial production and plating of conidia following mutagenesis and the procedures for sampling and genotype testing of survivors and storage of cultures were all as previously described (Simpson & Caten, 1979). The media for penicillin fermentation and biological assay were those described by Macdonald *et al.* (1963) and by Merrick & Caten (1975*a*, *b*), respectively. To compensate for a pleiotropic effect of the *pabaA1* mutation on penicillin titre, the fermentation medium was supplemented with *p*-aminobenzoic acid to 2 μg ml^{-1}. With the exception of the penicillin fermentations, which were carried out at 25 °C, all incubation was at 35 °C.

Antibiotic yield testing. The penicillin fermentation and biological assay were as described by Merrick & Caten (1975*a*, *b*) and subsequently modified by Simpson & Caten (1979). However, because the frequency of mutants with increased titre (Simpson & Caten, 1979) was lower than the frequency of comparable recombinants in crosses (Merrick & Caten, 1975*b*), the size of the population screened was increased to 100. This necessitated the following modifications to the experimental design. Each strain was fermented in a single shake-flask from which two samples were taken for assay. These were assigned to two separate assay 'blocks', each of which consisted of two 8 × 8 assay plates (i.e. 128 wells per block) over which the 100 test strains were randomly distributed (50 per plate). Five flasks of the parental strain (7/142 or 7/151 as appropriate) were also fermented in each cycle as controls. A sample from each flask of the control strain was assayed on both plates of each assay block. The remaining nine wells on each plate were taken up by three replicate benzyl penicillin standards at 2·0, 1·0 and 0·5 u ml^{-1}.

Selection programmes. Two comparable, but independent, recurrent mutation and selection programmes, each of six cycles, were carried out. For programme A, strain 7/142 was used as the initial parental strain and 8-methoxypsoralen in conjunction with near-ultraviolet light (8MOP) as the sole mutagen. For programme B, the parent strain was 7/151 and ethyl methanesulphonate (EMS) was used as the sole mutagen. The conditions of mutagenesis were as previously described (Simpson & Caten, 1979) and the treatment times were chosen to give approximately 10 % survival, being 40 min for EMS and 15 min for 8MOP. These conditions and treatment times were kept constant throughout all cycles of mutagenesis.

For the initial cycle of each programme, conidial suspensions of 7/142 (programme A) and 7/151 (programme B) were treated with 8MOP and EMS, respectively. Following mutagenesis, random samples of 50 and 100 survivors were picked from 7/142 and 7/151, respectively, ignoring colony morphology. The survivors were assayed, their titres were inspected and the two and five descendants with the highest titres were retained from 7/142 and 7/151, respectively, while the rest were discarded. These seven strains, and the comparable strains in subsequent cycles, were checked for retention of the parental markers and for heterokaryon compatibility group (Merrick, 1975*a*) to guard against the inclusion of revertants or contaminants in the selection programmes. This completed the first cycle. To prepare cell populations for the second cycle of mutagenesis, the selected strains were independently subcultured and a conidial suspension (10^{7} ml^{-1}) of each was prepared. Samples (3 ml) of the suspensions within each programme were pooled and the two final suspensions were exposed to mutagenic treatment either undiluted (descendants of 7/151, EMS) or following dilution to 10^{6} conidia ml^{-1} (descendants of 7/142, 8MOP). This method has the advantage of being technically simpler than separate mutagenesis of each selected strain but does not allow for differential susceptibility to the mutagen. Thus, the population of survivors may not contain equal frequencies of derivatives from the selected parent strains. In the second and all subsequent cycles of both programmes, 100 survivors were picked for assay and the five highest producers were retained. The conidial suspensions for each subsequent cycle of mutagenesis were prepared in the manner used for the second cycle.

Six successive cycles of mutagenesis and selection were carried out in each programme. Each cycle was given a code number, those in programme A being numbered A1 to A6, and those in programme B being numbered B1 to B6. Although both programmes were run concurrently each population of strains (12 altogether) was fermented in a separate experiment. All strains assayed were identified by the cycle code followed by a number denoting the order in which they were isolated, e.g. A1-1, A1-2 etc. The selected strains in each cycle were stored as dry conidia on silica gel at 4 °C (Roberts, 1969). For each experiment a standard analysis of variance (Simpson & Caten, 1979) was carried out on the $\log_{10}$ titres of the population of test strains and the following statistics were calculated: population mean titre, the mean titre of the selected top 5 % strains, the variation between sister strains (σ_G^2) and the environmental variation (σ_E^2). The mean titre of the five replicate flasks of the control strain (7/142 or 7/151 as appropriate) was also calculated.

Comparison of successive cycles. In any selection programme there is a need to make comparisons between successive cycles in order to determine whether a genuine response has been achieved. A problem arises, however, because successive cycles involve different experiments run on different occasions and any trend may be obscured by random environmental variation. In an attempt to overcome this difficulty, Merrick (1975*a*, *b*)

Table 1. *Selected strains from each cycle of recurrent mutation and selection in programmes A and B*

With the exception of experiment A1, five strains were selected from the population of 100 in each cycle of each programme.

Cycle of selection	Programme A	Programme B
1	A1:5, 32	B1:1, 2, 47, 66, 97
2	A2:28, 45, 61, 102,*	B2:5, 13, 29, 37, 66
3	A3:1, 32, 54, 63, 92	B3:1, 2, 5, 15, 26
4	A4:3, 38, 58, 87, 99	B4:1, 44, 50, 93, 100
5	A5:1, 12, 44, 71, 76	B5:27, 66, 68, 71, 84
6	A6:9, 40, 56, 63, 73	B6:21, 27, 56, 65, 73
Highest-titre strain	A6-9	B6-27

* One of the selected strains was shown to be a contaminant and discarded.

adopted two procedures – standardization against control strains and contemporaneous comparison of selected strains – both of which were used in the present selection programmes.

Merrick (1975*a*) included two control strains in each fermentation and standardized the mean of the test strains relative to these controls, to correct for the random environmental variation between occasions, by using the proportional relationship: $p = (m/m_x) \times p_x$, where p is the corrected mean titre of the test strains on occasion x, m is the mean of the control strains over all occasions, m_x is the observed mean of the control strains on occasion x and p_x is the observed mean of the test strains on occasion x. In the present selection programmes, 7/142 was included as a control in all six fermentations involving its derivatives (programme A) and 7/151 in all six fermentations involving its derivatives (programme B). The mean of the appropriate control strain over the six fermentations was used to estimate m. Fresh cultures of the control strains were taken from the silica gel stocks for each cycle. Therefore, it is reasonable to assume that these strains remained genetically constant throughout the entire selection programme and that any variation in their performance was due solely to environmental factors. The means of both the total test population and the selected top 5 % strains were standardized in this way. Throughout the programme the selected strains from each cycle were stored on silica gel. These strains are listed in Table 1. One of the five selected strains from cycle A2 was rejected as a contaminant and all results relating to this strain were excluded from subsequent calculations. At the end of the programme it was therefore possible to compare the titres of all the strains selected in one programme together with their unselected ancestor (7/142 or 7/151) in a single experiment. Separate experiments were carried out for the two programmes. Each of the selected strains from programmes A and B was fermented in three or two replicate flasks, respectively, with the replicate broths assigned to separate assay plates. Five replicate flasks of the appropriate ancestral strain were included in each experiment, with each flask assayed on each plate.

RESULTS

Comparison of successive cycles

Highly significant differences ($P < 0.001$) in penicillin titre between sister strains ($\sigma_G^2 > 0$) were detected in each cycle in each programme (Table 2). This confirmed the effectiveness of the mutagenic treatments since no such genetic component of variation had been detected in populations of untreated single conidial isolates of either parental strain (expts 1, 5 and 6 in Simpson & Caten, 1979). The presence of significant genetic variation in each cycle suggests that selection should be effective, provided that at least some of the variation is due to titre-increasing mutants. Examination of the standardized mean titres of the top 5% strains in successive cycles indicated a significant response to selection in each programme (Fig. 1). The population and top 5% means for populations of untreated single conidial isolates of 7/142 (programme A) and 7/151 (programme B) are included in Fig. 1 as cycle 0 to show the characteristics of the ancestral population. These data were obtained from experiments 5 and 6 and from experiment 1, respectively, of Simpson & Caten (1979). The mean of the top 5% strains increased from 6·9 to 18·0 u ml^{-1} (Fig. 1*a*) in programme A, and from 3·6 to 9·3 u ml^{-1} in programme B (Fig. 1*b*). Thus, about threefold increases in penicillin titre were achieved in each selection line during six cycles of recurrent mutation

Table 2. *Estimates of the variation in penicillin titre (log u ml^{-1}) attributable to environmental (σ_E^2) and genetic (σ_G^2) factors amongst populations of strains from successive cycles of programmes A and B*

Cycle of selection	Programme A σ_E^2	Programme A σ_G^2	Programme B σ_E^2	Programme B σ_G^2
1	0·004	0·005*	0·002	0·089*
2	0·044	0·055*	0·003	0·011*
3	0·003	0·025*	0·008	0·037*
4	0·008	0·184*	0·002	0·025*
5	0·004	0·030*	0·005	0·011*
6	0·015	0·040*	0·006	0·044*

* Significantly greater than zero, $P < 0·001$.

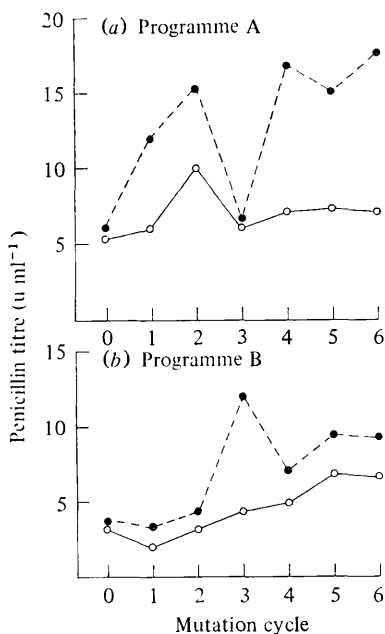

Fig. 1

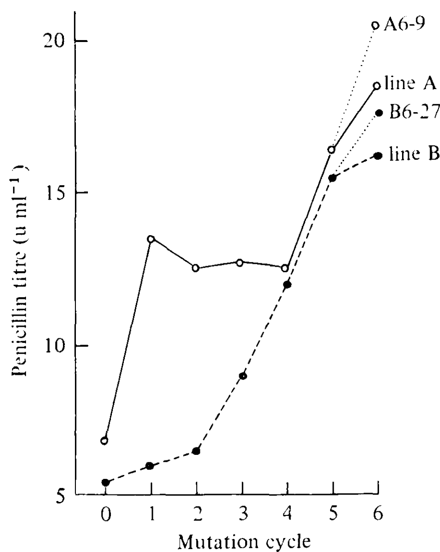

Fig. 2

Fig. 1. The population mean titre (○) and mean titre of the selected strains (●) in successive cycles of selection for increased penicillin titre in programmes A (*a*) and B (*b*).

Fig. 2. The mean titres in the contemporaneous comparison of the ancestral strain (cycle 0) and of the selected strains (cycles 1 to 6) for programmes A and B. A6-9 and B6-27 are the highest-titre strains recovered in each programme.

and selection. In programme B, this increase in the performance of the top 5% strains was accompanied by a twofold increase in the mean titre of the entire population. Such a result would be expected as the population in any cycle was derived solely from the top 5% strains of the previous cycle. The failure to detect a similar increase in the population mean for programme A is therefore surprising and suggests the induction of a large number of titre-decreasing mutations in each of the later cycles of this programme. These would depress the population mean, but would not be reflected in the performance of the selected top 5% strains.

The environmental variation (σ_E^2) varied significantly from experiment to experiment (both programmes, $P<0·001$) but there was no obvious trend (Table 2). Instead, this

Table 3. *Analyses of variance for penicillin titre (log u ml^{-1}) in the contemporaneous comparison of selected strains from six cycles of mutation and selection for programmes A and B*

	Degrees of freedom	Mean square	Variance ratio	*P*
Programme A				
Between strains	26	0·080	6·2	< 0·001
Between cycles	6	0·282	17·6*	< 0·001
Within cycles	20	0·016	1·2	NS
Between plates	2	0·004	0·3	NS
Strains × plates	52	0·013		
Total	80			
Programme B				
Between strains	30	0·090	12·9	< 0·001
Between cycles	6	0·374	19·7*	< 0·001
Within cycles	24	0·019	2·7	0·01
Between plates	1	0·112	16·0	< 0·001
Strains × plates	30	0·007		
Total	61			

NS, Not significant.
* Tested against strains within cycles mean square.

variation could be attributed to the occasional 'rogue' assay, which gave much poorer agreement between replicates than normal (e.g. experiments A2, B3).

Contemporaneous comparison of selected strains

The mean titres in the contemporaneous comparison of the selected strains in each cycle are plotted in Fig. 2. In both selection programmes, a near threefold increase in productivity over the six cycles was revealed, confirming the conclusions from the comparison of successive cycles. While small, steady increases were obtained in each cycle of programme B, the response in programme A occurred entirely in cycles 1, 5 and 6. Cycle A1 produced more than a doubling in titre suggesting the selection of two major mutants (only two strains selected in this cycle). The individual strains in each selection programme with the highest titres in the contemporaneous comparison were A6-9 and B6-27 with yields of 20·5 u ml^{-1} and 17·6 u ml^{-1}, respectively.

An analysis of variance of the log titres obtained in the contemporaneous comparison showed that in both selection programmes the differences between the selected strains were highly significant (Table 3). This variation was largely attributable to differences between selection cycles, thus confirming the selection responses. No significant difference between strains within selection cycles was found in programme A, indicating that the titres of the selected strains within each cycle were similar and, with the exception of cycle A1, the response observed was not due solely to the selection of one or two improved strains at each cycle. However, a significant difference was detected between strains within selection cycles for programme B, suggesting that at some cycles the response was largely attributable to the improved performance of only one or two of the selected strains.

DISCUSSION

Following mutagenesis, variants with small increases in the productivity of secondary metabolites occur far more frequently than those with large increases (Brown & Elander, 1966). It is likely therefore that more rapid advances in yield could be achieved through the accumulation of small improvements over successive cycles of mutagenesis than by screening for the rare major mutants in large populations from single mutagenic treatments (Davies,

1964). A problem arises, however, through the large testing errors inherent in fermentation and assay procedures which make the unambiguous identification of individual strains with small increases difficult. A way round this difficulty adopted in the past is to use multi-stage yield tests (Backus & Stauffer, 1955; Alikhanian, 1962). These, however, entail heavy investment of labour and facilities at the testing stage and hence reduce the number of strains that can be screened. An alternative procedure was suggested by Davies (1964) on the basis of results from computer simulations of mutation and selection programmes. These indicated that the rate of improvement could be increased by adopting a process of mass selection in which the top few percent of strains in each cycle are carried forward rather than just a single strain, thereby reducing the need for repeated testing. In addition, since several strains are carried forward, mass selection offers some protection against picking a strain that is resistant to further mutagenesis or that suffers from a scale-up defect. While the procedures suggested by Davies have undoubtedly been widely adopted by industry, we have been unable to find any published reports of strain development in which they have been employed. The results of the present programmes, where the top 5% strains in each cycle were selected on the basis of a single-stage screen, are therefore of interest in testing the application of the conclusions from computer simulations.

In both programmes, a near 300% increase in penicillin titre was obtained over six cycles of mutation and selection (Fig. 2). In each programme approximately 600 strains were tested separately in single shake-flasks. Comparisons with strain improvement programmes employing different screening and selection procedures are difficult both because different organisms, products and mutagens are involved and because detailed information concerning the number of strains screened and shake-flask tests conducted is not always available. Backus & Stauffer (1955) achieved a fivefold increase in penicillin titre in strains of *Penicillium chrysogenum* over ten cycles and Dulaney (1953) increased the streptomycin titre of a strain of *Streptomyces griseus* from 250 u ml^{-1} to 2500 u ml^{-1} over eight cycles. Both these programmes, however, involved screening many more strains and carrying out many more yield tests than the present modest programme. A comparison is possible with the isolation of single mutants giving major increases in penicillin titre from the same or closely related strains of *A. nidulans* (Ditchburn *et al.*, 1976). Two mutants with a threefold and one mutant with a twofold increase in titre were isolated from 2800 strains screened. However, since many strains were tested on more than one occasion, the number of individual shake-flask tests considerably exceeded the number of strains. These comparisons suggest that in terms of the rate of advance per test, the mass selection procedure used in the present work was at least as efficient as the earlier procedures, in agreement with the conclusion from Davies' simulation of strain development programmes.

Merrick (1975*a*, *b*) selected for increased penicillin titre among the progenies of sexual crosses between unrelated wild-type isolates of *A. nidulans*. The parents were chosen from a collection of isolates for their high titre and for their membership of different heterokaryon compatibility groups. The latter condition was intended to maximize the genetic differences between the parent isolates (Merrick & Caten, 1975*b*), in contrast to the present experiments where the two initial strains belonged to the same compatibility group and had been repeatedly backcrossed to increase their isogenicity (Simpson & Caten, 1979). Fermentation conditions, assay procedures and selection intensity were all similar to those used here. Although the hybridization programmes achieved higher titres, the proportional increases were less than in the present mutation programmes since the initial titres were higher, despite the fact that overall more cycles and tests were involved (Merrick, 1975*a*, *b*). The major factor limiting the response was the amount of variation for penicillin titre present in the natural isolates. The achievement of larger proportional increases in the present mutation programmes therefore clearly demonstrates that even modest mutation programmes generate more useful variation in secondary metabolite production than occurs in natural populations of the producing organism. This is in agreement with the general experience that the screening

of natural isolates for increased potency is only of value during the initial stages of a strain development programme (Alikhanian, 1962; Calam, 1964).

There were large fluctuations between successive cycles for both the population mean and the mean of the top 5% strains (Fig. 1). These may result from four factors. Firstly, the failure of the standardization procedure to correct fully for the environmental effects between fermentations. Secondly, because of the low level of replication of individual strains, the mean of the top 5% strains will be considerably influenced by testing error. Thirdly, the composition of the population assayed in each cycle will be affected by the sampling of the survivors. Finally, the mutagenic treatments, although kept constant, may have had different effects in different cycles. The contemporaneous comparison of selected strains removed most of the random fluctuation from cycle to cycle (Fig. 2). This suggests that environmental differences between fermentations were the most important factors generating fluctuation and therefore that the standardization procedure had not been effective in correcting for this variation. Why the standardization should have failed is not clear, but it is possible that the replication of the control strains was insufficient to allow for the effects of the testing errors on the control means.

The two selection programmes led to the production from the unselected parents 7/142 (6·8 u ml^{-1}) and 7/151 (5·5 u ml^{-1}) of two independent improved strains A6-9 (20·5 u ml^{-1}) and B6-27 (17·6 u ml^{-1}), respectively (titres from contemporaneous comparison). This response to selection for increased penicillin titre confirms the conclusion from the statistical analysis of single mutagenized populations that treatment with 8MOP or EMS to 10% survival produces a significant frequency of titre-increasing mutants (Simpson & Caten, 1979). The improved strains A6-9 and B6-27 derived in this way have been used for a biometrical analysis of the genetic changes underlying the increased productivity in each selection line and of the combining ability of the mutations induced in the two separate selection lines.

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