

VARIATION AND MUTATION IN *PENICILLIUM* *CHRYSOGENUM*, WIS. Q176

ELWYN REESE, KATHRYN SANDERSON, ROLAND WOODWARD, AND
G. M. EISENBERG

J. T. Baker Chemical Company, Phillipsburg, New Jersey

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In May, 1946, a project of strain development was undertaken with the primary objective of obtaining increased yields of penicillin. Other objectives included the possibility of reducing pigment formation, of obtaining different types of penicillin, and of determining the stability of this strain in culture. With the facilities available it was not possible to devote any time to the second of these. It is the purpose of this paper to present a brief report of the results obtained.

Raper (1947) summarized the history of *Penicillium chrysogenum*, Wis. Q176, the strain currently in use in commercial production of penicillin, as follows:

NRRL 1951—*P. chrysogenum*, isolated from a moldy cantaloupe, capable of producing approximately 100 u per ml of penicillin in submerged culture.

NRRL 1951.B25—A naturally occurring variant from NRRL 1951, capable of producing up to 250 u per ml of penicillin.

X1612—An X-ray-induced mutation from NRRL 1951.B25, capable of producing more than 500 u per ml of penicillin.

Wis. Q176—An ultraviolet-induced mutation from X1612, capable of producing more than 900 u per ml of penicillin.

The importance of the foregoing developments to present penicillin production cannot be overemphasized, because current yields of 750 to 900 units per milliliter are obtained in nutrient solutions of approximately the same composition as those used to produce maximum yields of 75 to 100 units per milliliter with NRRL 832 just a short time ago.

It is apparent that great increases in yields have been obtained by the production of mutants, based on the evaluation of these in shaker flasks in the laboratory. From the industrial standpoint, the practical questions arise (a) whether further increases may be expected, and (b) whether the increased yields will be sufficient to justify the expenditure. There is no a priori reason for believing that strain Q176 is the best possible. The fact that the last mutant resulted in a doubling of yield would indicate that an end point has not been reached, since it appears probable that the increments (of increase) would become progressively smaller as the yield of penicillin approached a maximum.

METHODS

Two means of producing mutants were employed in this study. Nitrogen mustard (methyl-bis(β -chloroethyl)amine HCl¹) was used first because (a) it

¹ Nitrogen mustard obtained through the courtesy of the Committee on Growth of the National Research Council.

was found by Stahmann and Stauffer (1947) to give a greater percentage of mutants, when the majority of the spores were killed, than ultraviolet radiation of 2,750 Å, and (b) because we believed the use of an agent different from those previously used in developing Q176 might prove more promising. A procedure suggested by Tatum (1946) was followed. To 5 ml of a concentrated spore suspension was added 5 ml of a 0.24 per cent solution of nitrogen mustard in citrate-phosphate buffer (pH 6.3; 0.12 M). Sterilization was accomplished by means of filtration through a sintered glass disk prior to the addition of spores. The control solution was prepared similarly, omitting only the N-mustard. One-ml aliquots were removed at different times and diluted to an appropriate range for plating out (1 ml flooded over the surface of a potato lactose agar plate). After 3 to 5 days' incubation at 25 to 30 C single colonies were removed from plates showing over 90 per cent kill and transferred to slants of potato lactose agar.

For the later work, ultraviolet irradiation was used exclusively as the means of inducing mutation. The spore suspension was contained in a quartz flask shaken at 6 inches above the "sterile-lamp" (WL 782-30). The ultraviolet output of the lamp was checked at each usage by means of a Westinghouse SM-600 U.V. indicating meter. Under similar circumstances, 90 per cent of the spores are killed in 10 minutes and over 95 per cent in 12 minutes. Survivors are then treated in the same way as those surviving the N-mustard exposure.

As the fermentation medium, the following was selected because of the rapid growth obtained, and because the potencies remained at a high level: cottonseed meal 3 per cent, lactose 2 per cent, glucose 0.1 per cent, phenylacetamide 0.1 per cent, NaNO_3 0.5 per cent, KH_2PO_4 0.5 per cent, MgSO_4 0.5 per cent, and chalk 0.5 per cent. In general, on cottonseed meal medium, peak potency was reached by the fourth day and continued at a high level for several days. In corn steep medium, the rise in potency is much slower, and the length of time that the potency remains at the peak is short. In order to obtain maximum potencies with greatest accuracy and to be able to set up a regular 7-day schedule, we selected the cottonseed meal medium.

One ml of spore inoculum was added to 110 ml of medium in a 1-liter Erlenmeyer flask. Two flasks were used for each strain. Incubation was at 24 C on a reciprocating shaker having a 4-inch stroke and 90 strokes per minute. Growth was noticeable in 2 days, and high potencies were obtained in 4 days. Assays were made on the fourth, fifth, and seventh days. Two sets of controls of Q176 of different histories were included in each test, with 50 test strains in duplicate. Strains were evaluated in terms of the control, those surpassing the highest control by 20 per cent being saved for retesting. The assays were run against *Bacillus subtilis* as the test organism.

RESULTS

The results are summarized in table 1. Each strain is evaluated in terms of the Wis. Q176 parent. This Q176 control usually reached a peak of about 450 u per ml under the conditions of the test. Five "yield groups" were set up as follows:

- A—0 to 40 per cent of yield of Q176.
 B—40 to 80 per cent of yield of Q176.
 C—80 to 120 per cent of yield of Q176.
 D—120 to 160 per cent of yield of Q176.
 E—over 160 per cent of yield of Q176.

It is apparent from the results that *untreated* single spore isolates of Q176 show a rather large amount of variation in yield, only 62 per cent being in the C group. Treatment with N-mustard resulted in strains only 53 per cent of which fell in the C group, whereas the irradiation treatment further reduced the percentage of strains falling in this group to 41. Treatment with these mutagens thereby resulted in a spreading out of the distribution curve, with many more survivors appearing in the end groups (A, B, D, E). Ultraviolet irradiation was the more severe treatment as judged by (1) the greatest reduction in the C group, (2) the largest percentage of survivors giving zero units of penicillin, and (3) the production of strains showing the highest yields. Nitrogen mustard appears to have been less severe, judged by these criteria, but, whereas with irradiation

TABLE 1
Summary of strains evaluated

TREATMENT	YIELD IN % OF Q176 CONTROL		NUMBER OF STRAINS TESTED						SAVED AFTER RETESTING*	
			A		B	C	D	E	Total	No.
	0%	1-40%	40-80%	80-120%	120-160%	160%				
Untreated single spore isolates of Q176.....	0	5	38	78	4	0	125	1	0.8	
N-mustard.....	5	29	182	329	64	9	618	20	3.2	
Ultraviolet.....	27	139	579	598	101	23	1,467	30	2.0	

* Those "saved after retesting" have been retested at least three times and average more than 15 per cent above the controls.

the curve shifted toward lower-yielding strains, with N-mustard the shift is toward higher-yielding survivors. An examination of the population of the high-yielding groups (D and E) reveals that the percentage of survivors in these groups are as follows: control, 3; N-mustard, 11.5; and ultraviolet 8.5 per cent.

Since these figures are based on single tests, they are subject to errors of various sorts associated with the mass handling of cultures, dilutions, and assays. A more valid result would seem to be based on the strains which after two or more retests still fall into groups D and E. The percentage figures for these strains are: control, 0.8; N-mustard, 3.2; and ultraviolet 2.0 per cent.

In terms of altered strains, i.e., those removed from the C group, the shift to D and E groups has been:

$$\frac{\% \text{ in D and E groups, treated} - \% \text{ in D and E groups, control}}{\% \text{ in C group, control} - \% \text{ in C group, treated}} \times 100$$

$$\text{N-mustard} = \frac{8.5}{62-53} \times 100 = 94\%$$

$$\text{Ultraviolet} = \frac{5.5}{62-41} \times 100 = 26\%$$

Such figures as these indicate great superiority of the N-mustard treatment in shifting the altered strains toward higher yields, with the irradiation shift being toward lower yields. In searching for high-yielding strains, however, *degree* of superiority is more important. An analysis of the 51 superior strains follows:

NUMBER OF STRAINS (OUT OF 2,200)	CONTROL	N-M.	U.V.
Yielding 15-35% above Q176.....	1	19	9
Yielding 36-55% above Q176.....	0	1	15
Yielding 56-75% above Q176.....	0	0	6

The ultraviolet-irradiated strains have been retested less frequently than the others, and a few may fall into a lower category. But allowing for this, it is obvious that the best strains are those resulting from ultraviolet irradiation (as were also the poorest).

The rate of appearance of high-yielding strains among the survivors of mutation-inducing treatments is rather high, i.e., of the order of 2 to 3 per cent. The rate of mutation is a function of the severity of the treatment, the degree of which is measured by the percentage killed. In our tests, the figures are:

N-mustard	99+% kill in 4 tests
	92% kill in 1 test
	84% kill in 1 test
Irradiation	99+% kill in 4 tests
	90-99% kill in 4 tests
	60% kill in 1 test

Our data are too limited to show any correlation between the percentage of survivors and the percentage of superior strains obtained.

The rate of mutation (based on mutants as percentage of survivors) obtained from irradiation, as compared to N-mustard treatment, is at variance with the findings of Stahmann and Stauffer (1947) working with a different mold strain of *P. chrysogenum* (NRRL 832). Using morphological differences as a criterion, Stahmann and Stauffer were able to obtain much more data. Their results show much higher mutation rates among the N-mustard survivors when the majority of the spores were killed. In accounting for the discrepancy between results, it should be indicated that our technique for using the N-mustard was different from theirs. It is possible that for the same percentage of killing with N-mustard, different mutation rates among the survivors exist which depend upon other environmental conditions. Experiments on the production of morphological mutants are necessary to test the validity of this hypothesis. A further difference in methods existed in that we used ultraviolet light of 2,537 Å whereas Stahmann and Stauffer used 2,750 Å.

Morphological mutants appeared in roughly the same numbers as biochemical (yield) mutants in the work here reported. The absence of pigment production seemed important enough to warrant some attention, since it seemed to us that a

whiter final penicillin might be more easily obtained from a strain producing little or none of the yellow pigment in the broth. Many such strains were found, but all except two of these gave low yields. (Stauffer, 1948, has just informed us that his group has recently released to industry a nonpigmented strain that performed as well as Wis. Q176 in their trials.) At the opposite extreme is a strain that produces a deeper yellow pigment in agar than any of the others, but this was not evident in broth. Another strain, which on agar is identical with Q176, in broth produces a dark brown culture unlike anything we had observed before. In both the latter strains the pigment seems to be without effect on the potency, each strain being normal in that respect.

Variation in sporulation was common. Under the conditions of growth (23 to 25 C on potato lactose agar), the usual response was light sporulation within a week to 10 days, the surface becoming light blue-green with varying amounts of yellow. Variations occurred in both directions, i.e., some strains were yellow, some white with no sporulation, and others were a velvety blue-green with dense sporulation. In general the abnormal sporeformers had a tendency to lower yields. One odd culture has tan spores that are particularly obvious when observed on Moyer's agar. This strain produced somewhat less penicillin than the control.

Natural Variation in P. chrysogenum, Wis. Q176

A matter of primary concern in industrial fermentations is the stability of the fungus culture. Great embarrassment and financial loss may result from variation in the organism. Raper (1946) and others claim *P. chrysogenum*, Wis. Q176, to be unstable, tending to produce morphological and biochemical variants. As a result, it is general practice to preserve the spores from an early transfer of the organism in soil tubes, or in lyophile vials. In this way variation is reduced to a minimum.

How variable is strain Q176? The evaluation of cultures from untreated single spores of Q176 (above) shows a distribution in which 62 per cent gave average yields, 33 per cent gave yields 20 to 60 per cent above or below average, and 4 per cent gave yields 60 per cent or more from the average. By average here is meant the behavior of the whole population from which the single spore isolates were made. It appears that the variation from single spores is great, and that the possibility of an inferior strain developing on repeated transferring ought to be guarded against.

Several series of transfers of strain Q176 were made to determine the rate of appearance of variants. The potato lactose agar slants were incubated at 23 to 25 C and transfers made when convenient (usually 4 to 14 days). The original transfers (20Y) were made at 30 C, at which temperature less sporulation occurs, and the first variant was a nearly white (20W) sector. These two cultures appeared identical when grown at 25 C. Ninety successive transfers of 20Y, over a 2-year period, have been made during which time no morphological variant has appeared, nor has there been any loss in penicillin-producing ability, as determined in shaker flask tests. On the 41st transfer of 20W, a heavily

sporulating (blue-green) sector (20W-S) appeared. No other variants appeared in 20W through a total of 90 transfers. This culture, like 20Y, retained its appearance and penicillin-producing ability. Variant 20W-S in addition to its more rapid sporulation differs in that less pigment is produced in the agar. In yield it is in a class with its parent 20W. Through 49 transfers it has retained these characteristics.

The foregoing transfers were made by removing bits of agar containing mycelium from a colony to a fresh slant. It later appeared that the nature of the medium might affect the rate of variation, or that an increase in variants might occur if spores only were used in the transfer. For checking the latter, a loop of spores was taken from a culture that sporulates well on Moyer's agar² and transferred to a fresh slant. During a series of 47 transfers on this medium, no variants were observed and no loss in penicillin-producing ability occurred. Moyer's agar is a high-salt agar used in producing spores of Q176 on which but little mycelium is produced. Variants are undoubtedly present among the spores transferred. But since no change has occurred, it appears that such variants as have been present have been less adaptable to the medium than are the "normal" spores, and as a result are suppressed. In this regard, Raper has written us, "Variants do not appear on rich media such as Moyer's sporulation agar with the frequency or prominence that they do on media such as Czapek's solution agar."

Nutrient glucose agar (Difco nutrient agar plus 0.2 per cent glucose) was used for a series of transfers. On this agar vegetative growth occurs, but sporulation is almost completely suppressed. As a result, the surface is white with no traces of blue, green, or yellow, but with an occasional brown tint in the center of old colonies. In 47 transfers no variation was noted, and no loss in penicillin-producing ability occurred. Transfers here, as in the cases to follow, were by pieces of agar containing mycelium.

Potato glucose (1 per cent) agar, with strain 20PD, results in more rapid growth than does potato lactose agar, and in earlier and better sporulation. On the fourteenth transfer, a white sector appeared (20PD-S). No other sectors appeared in the remaining 33 transfers, nor was there any loss in penicillin production. The white sector (20PD-S), on microscopic examination, showed good development of sporophores, but only an occasional spore. Transfers to Moyer's agar of all our other cultures resulted in normal, heavy, blue-green sporulation. This sector from potato glucose agar (20PD-S) when grown on Moyer's agar is a much lighter blue-green, but spores do develop. The capacity of this strain to produce penicillin is decidedly less than that of the other cultures (by about 50 per cent). No change in this culture (20PD-S) in 33 transfers was noticed.

These observations may be summed up as follows: In 403 transfers, two morphological variants were found. Of these, only one resulted in a loss in ability to produce as much penicillin as the parent in shaker flask tests. Apparently

² Moyer's agar as modified for our work: glycerol, 2.0 g; Brer Rabbit molasses, 2.0 g; curbay B.G., 2.5 g; peptone, 2.0 g; MgSO₄·7H₂O, 0.05 g; KH₂PO₄, 0.06 g; NaCl, 40 g; lactose, 20 g; agar, 25 g; water to 1 liter.

no variant that was morphologically identical with the parent but different in ability to produce penicillin appeared. The data are too limited to make any claims for superiority of medium, though our personal preference is for potato lactose agar. It must be emphasized that the conditions under which these transfers were made minimize the possibility for variation. Higher temperatures of incubation and longer periods between transfers would unquestionably have resulted in greater variation. It appears from these data that *P. chrysogenum*, Wis. Q176, can be maintained in a satisfactory condition for fermentation by transfers on agar made at weekly intervals and incubated at 23 to 25 C, provided that a little attention is paid to the appearance of sectors.

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SUMMARY

Several mutants of *Penicillium chrysogenum*, Wis. Q176, have been produced which in shaker tests surpass the parent in yields of penicillin by at least 50 per cent.

Ultraviolet irradiation resulted in the production of greater numbers of variants than did N-mustard treatment.

P. chrysogenum, Wis. Q176, can be maintained by serial weekly transfers on agar slants without loss in penicillin-producing capacity.

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