MUTANTS RECOVERED AFTER EXPOSURE OF STREPTOMYCES VENEZUELAE TO X-RAYS

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Received for publication July 29, 1955

There have been relatively few studies of induced mutations in actinomycetes when compared with certain bacteria and fungi. Most of these studies (Dulaney et al., 1949; Katagiri, 1954; Okami and Umezawa, 1950; Okami et al., 1950; Savage, 1949) were concerned primarily with obtaining strains with enhanced antibiotic production, although a few (Appleby, 1948; Kelner, 1948; Newcombe, 1953) dealt with other types of mutants. None of these studies included Streptomyces venezuelae, the actinomycete which produces chloramphenicol. Our study on the irradiation of spores of this species with X-rays was aimed at (1) determination of the relationship between dosage and the survival of this organism and a quantitative estimation of the most readily detected mutants at various dosages and (2) isolation of mutants with blocks in the reactions leading to chloramphenicol for the purpose of elucidating the biosynthesis of this antibiotic (Gottlieb et al., 1954). This paper, a preliminary report of which has been presented (Gwatkin and Gottlieb, 1955), deals primarily with the first of these considerations1.

MATERIALS AND METHODS

S. venezuelae strain R-1 was grown on tryptone-glycerine agar (tryptone, 5 g; glycerine, 10 g; NaCl, 5 g; BY* extract, 100 ml; distilled water, 900 ml) in flat bottles for 11 days at 26 C. These cultures were stored at 5 C and served as a source of spores throughout the study.

When required for irradiation, the spores were washed from a culture by adding a sterile solution of 1 part "vatsol"3 to 10,000 parts of distilled water and agitating gently. The resulting suspension was placed in a Waring blender for 8 min and then filtered through no. 2 Whatman filter paper. More than 90 per cent of the particles in a filtered suspension were single conidia, as determined by microscopic examination. Such spore suspensions were brought to a turbidity (O.D., 27.0 at 530 mp) corresponding to approximately 1016 viable spores per ml, as determined by plating out on tryptone-glycerine agar.

Three ml of a spore suspension were irradiated in a 15-ml beaker covered by a piece of dialyzing membrane. Because the depth of the suspension was only 2 to 3 mm, the X-ray dosage could be considered uniform throughout, so that no stirring device was necessary. Several of these 3-ml samples were transported to the X-ray apparatus on ice, one sample being used for each X-ray dose.

The source of X-rays was a high intensity beryllium window tube with a tungsten target which delivered unfiltered X-rays. The intensity of these rays at the surface of the sample, 14 cm from the target, was 50 r per min, at 40 kv and 13 ma. Dosages ranged from 25 to 300 kr.

Following irradiation, spore suspensions were usually stored overnight at 5 C. Samples were then plated out in triplicate on tryptone-glycerine agar, the plates incubated at 26 C and colonies counted 48 hr later to determine survivors. With this information dilutions were prepared from the irradiated suspensions so that, when 0.1-ml amounts were spread on agar plates, 5 to 10 colonies developed on each plate. The purpose of spreading rather than pouring plates was to insure surface growth of colonies so that any variations in colony morphology would readily be observed. The plates were incubated 4 days at

1 This work was supported in part by National Institutes of Health Grant G-4118.

* To prepare the extract, 50 g of BY (fermentation solubles obtained from Commercial Solvents Corp., Terre Haute, Indiana) were added to approximately 900 ml of distilled water, the mixture boiled 3/2 hr, filtered, and the filtrate was made up to 1 L.

2 Vatsol is a detergent produced by American Cyanamid Co., Agricultural Division.
26 C, then sprayed with Bacillus subtilis, using a modification of a spraying apparatus designed by Stansley (1947), and reincubated overnight at 37 C in order to detect those colonies not forming appreciable amounts of chloramphenicol. Growth of B. subtilis in no way interfered with observations of morphological and other changes in the colonies. When pure cultures of mutant colonies were desired, transfers were made to tryptone-glycerine agar containing 20 μg chloramphenicol per ml to free the cultures of B. subtilis.

RESULTS

Survivors. At 25 kr there were about 10 per cent survivors. As the dosage of X-rays was increased to 100 kr, the number of survivors dropped to about 0.1 per cent. The rate of killing decreased with increasing dosage up to about 100 kr. Above this dosage the rate of killing appeared to remain constant up to 300 kr, the highest dosage given. The relationship between dosage and the survival of S. venezuelae is shown in figure 1. The curve represents the average of four independent experiments.

Mutant types. On tryptone-glycerine agar plates, parent or wild type colonies were 3 to 5 mm in diameter, with smooth margins, gray-white sporulating upper surfaces, and appeared yellow-brown when viewed from below. A brown melaninlike pigment, typical of the so-called chromogenic actinomycetes, was present in the medium around these colonies. Readily distinguishable from the parent colonies were the following mutant types:

1. Asporogenous mutant colonies which lacked spore-bearing aerial mycelium.
2. Yellow mutant colonies which lacked the brown melaninlike pigment and appeared yellow in color.
3. Irregular mutant colonies which were somewhat reduced in size, flattened, and had irregular colony margins.
4. Dwarf mutant colonies in which the diameter of the colony was reduced to 0.5 mm or less. Dwarf colonies always lacked aerial spores and were usually brown pigmented.
5. Colonies without zones of inhibition which represented low or non-producers of chloramphenicol. Such colonies were either indistinguishable in other respects from the parent type or they showed, in addition to altered chloramphenicol production, characteristics of any one of the above mutant types.

Some of these mutant types are shown in figure 2. Colonies differing in other ways from the parent type were also noted, e.g., colonies with partially reduced sporulation and colonies with slight

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**Figure 1.** Relationship between X-ray dosage and the survival of Streptomyces venezuelae.

**Figure 2.** Mutant colonies on tryptone-glycerine agar plates sprayed with Bacillus subtilis. **Upper right:** chloramphenicol-producing dwarf colony. **Upper left:** irregular colony not producing chloramphenicol. **Below:** 3 chloramphenicol-producing parent colonies flanked on either side by 2 asporogenous, irregular, yellow mutants which have produced less chloramphenicol than the parent.
variations in color or morphology. Because of the difficulty in differentiating these from the parent, they were counted with the parent colonies when frequencies of mutants were determined.

Several mutant characteristics were often associated. Thus, it was rare to find a yellow mutant that was not also irregular. Asporogenous colonies were usually both irregular and lacking in brown pigment.

Yellow, irregular, and dwarf mutants represented less than 1 per cent of the colonies which developed from nonirradiated spore suspensions.

Asporogenous mutants and colonies without zones of inhibition represented 3 and 13 per cent of the colonies respectively. On irradiation, the frequency of all the mutants was increased. Nearly maximum frequencies were obtained with 50 kr; higher dosages did not give appreciably greater frequencies. Figure 3 shows the dosage-frequency curves of the various mutant types.

Despite the fact that, at maximum frequency, 38 per cent of the colonies did not inhibit B. subtilis, only 2 per cent or less of these colonies gave rise to strains which did not produce detectable amounts of chloramphenicol in liquid shaken culture. A number of these nonproducing strains are being tested for the accumulation of suspected intermediates.

Stability of mutants. When asporogenous and yellow mutant colonies were subcultured onto tryptone-glycerine slants, some lost their mutant characters and reverted to the parent type. On slants the dwarf mutants gave rise to bacteria-like cultures: opalescent, soft, and usually brown pigmented. Figure 4 shows the appearance of slant cultures of these mutants. The colony irregularity characterizing the irregular group of mutants could not be detected in the confluent growth which occurred in slant cultures. Plating, however, revealed that in most cases, the feature of colony irregularity had been retained.

Stability of 93 mutants belonging to yellow, asporogenous, and dwarf types was tested by 6 weekly transfers on slants of tryptone-glycerine agar. Yellow and dwarf mutants were relatively stable. On the other hand, asporogenous mutants yielded relatively unstable populations, tending to regain sporulation. When asporogenous mutants belonged to the yellow type, this tendency of the progeny to regain sporulation was greatly reduced (table 1).

Fragmentation of the mycelium. Strains of dwarf and irregular mutants, unlike the parent or the other mutants, yielded cultures which were soft in consistency and with mycelia which fragmented very readily. This softness and tendency toward fragmentation of the mycelium was more pronounced in the dwarf mutants than in those of the irregular type. Microscopic examination of a large number of dwarf mutants in tryptone-glycerine shaken culture showed that mycelia with a few branches were formed during the early stages of growth, but that these later
TABLE 1

<table>
<thead>
<tr>
<th>Mutant Type</th>
<th>Number of Strains</th>
<th>Strain Stable after 6 Transfers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dwarf</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Asporogenous</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Yellow</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Asporogenous and yellow</td>
<td>34</td>
<td>18*</td>
</tr>
</tbody>
</table>

* The progeny of 18 mutants sporulated; only 2 produced a small amount of brown pigment.

break up to give cultures which consisted almost entirely of bacillary and coccoid fragments.

Nutritionally deficient mutants. The parent strain and all yellow and asporogenous mutants tested grew well on the synthetic medium of Gottlieb et al. (1954) in which only the carbon source is organic. Dwarf mutants, on the other hand, would not grow unless a supplement, 0.1 per cent yeast extract, was added to the medium. The growth factors required by the dwarf mutants appeared to be other than amino acids, since a supplement of 0.1 per cent Difco casamino-acids (vitamin-free) failed to support growth.

Acid production. After 4 days of incubation with shaking at 26 C in tryptone-glycerine, medium dwarf mutants lowered the reaction of the medium to pH 4.3 to 4.4. In contrast, the parent strain and the other mutants tested gave an alkaline reaction, typical of the majority of Streptomyces.

DISCUSSION

Forty-seven kr resulted in 1 per cent survivors (figure 1). One-third of this dose has been reported as producing the same mortality in S. flaveolus (Kelner, 1948) and one-eighteenth of this dose in the case of S. griseus (Savage, 1949). It seems unlikely, particularly in the case of S. griseus, that such a marked difference in sensitivity can be attributed solely to variations in irradiation technique.

It should be borne in mind that the techniques used did not reveal whether the increased frequency of mutants following irradiation was due primarily to mutagenic effects, selective killing, or postirradiation production of selective effects.

Kelner (1948) has reported curves for S. flaveolus relating X-ray dosage and mutant frequency which are similar to those given in this paper (figure 3). However, in S. flaveolus, higher dosages (200 kr or more) were required to give maximum frequencies.

Because of mycelial fragmentation and absence of spore-bearing aerial mycelium, all dwarf and some irregular mutants might be classified in the genus Nocardia (Berger's Manual of Determinative Bacteriology, 1948). In view of these mutant types, and considering another stable asporogenous variant observed in S. griseus (Appleby, 1948), one wonders how many of the Nocardia species represent Streptomyces which have permanently lost the ability to sporulate.

ACKNOWLEDGMENTS

The authors wish to thank Dr. G. L. Clarke, Department of Chemistry, University of Illinois, for use of the X-ray equipment. The studies reported in this paper were initiated in the Department of Horticulture, prior to the establishment of the Department of Plant Pathology. Use of the facilities of the Department of Horticulture is gratefully acknowledged.

SUMMARY

A chloramphenicol-producing strain of the actinomycete, Streptomyces venezuelae, was irradiated with X-rays to determine the general response of the organism to such treatment, and to obtain nonchloramphenicol-producing mutants which might accumulate intermediates in the biosynthesis of this antibiotic.

Readily recognized mutants were those involving loss of spore-bearing aerial mycelium (asporogenous), loss of brown melanin-like pigment (yellow), colony irregularity (irregular), diminution in colony size (dwarf), as well as loss of chloramphenicol production. The most frequent mutants were asporogenous and irregular, yellow, and dwarf in decreasing order. Dwarf mutants were nutritionally deficient and produced large amounts of acid. Although 38 per cent of the survivors formed colonies without zones of inhibition against B. subtilis, only 2 per cent or less of cultures from such colonies failed to produce detectable amounts of antibiotic in liquid shaken culture.

Yellow and dwarf mutants were found to be extremely stable. By contrast, the progeny of
asporogenous mutants were much more unstable, tending to regain spore-bearing aerial mycelium.

Mycelia of irregular and dwarf mutants fragmented to a much greater extent than the parent strain. This was especially marked in the dwarf mutants. The tendency for fragmentation, combined with the loss of sporulation, might place many of these mutants in the genus Nocardia.

REFERENCES


