ADVANCES IN

Applied Microbiology

Edited by WAYNE W. UMBREIT

VOLUME 4

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VOLUME 4

1962

ACADEMIC PRESS, New York and London

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, N. Y.

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
BERKELEY SQUARE HOUSE, LONDON W. 1

Library of Congress Catalog Card Number 59-13823

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

The continued expansion of *Advances in Applied Microbiology* and its gradually developing role as an authoritative information source give the editor a certain amount of support for his contention that the essay type of publication plays a unique role in modern science. This function is that of a seasoned guide through the jungle of contemporary publication. The present volume retains its international outlook and we shall do our best to obtain the best essayists possible, no matter where they may be located.

W. W. UMBREIT

Rutgers University
June, 1962

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I. Introduction

Mutagenic factors enhance microbial variation and substantially increase the rate of selection. Furthermore, microbial variation may be enhanced by phages as transmitters of genetic information. In recent years attempts have been made to use hybridization for this purpose, as well.

Up to the present time it was selection against the background of natural variation that served as means for increasing the productivity of valuable forms of microorganisms. For the last 15 years great importance has been attached to induced mutations, i.e., to breeding mutant clones of microorganisms by treating their parent forms with various physical and chemical factors.

The regularities of selection with the use of natural variation, change sharply when one begins to use mutagenic factors for enhancing variation. As is known the curve of selection by quantitative features goes up at the beginning of the work with wild (natural) forms of microorganisms. As the economically valuable feature is enhanced, the rate of this enhancement falls and at a certain level of the feature the selection gives no practical effect. The ascending curve of the feature enhancement shows a plateau.

To eliminate this plateau and to continue the enhancement of the valuable feature many laboratories of the world began to successfully use mutagenic factors. At the new stage in selection of microorganisms, which is characterized by a wide use of mutagenic factors, new problems pertaining to the development of principles and methods of using mutagens in the work with industrial microorganisms have arisen.

The regularities of natural selection are quite well studied in diploid and, to a lesser extent, in haploid organisms. If the regularities of selection in diploid organisms are "complicated" with absorption of newly developed changes due to the sexual process and recombinations pertinent to it, the selection in haploid organisms acts more graphically, i.e., more rigidly. All unfavorable mutations in haploid organisms are completely eliminated: a fact which greatly facilitates the selection of necessary forms.

The methods and principles of selection with the use of mutagenic factors are developed rather poorly, both in diploid and haploid organisms (molds, actinomycetes). The work on using mutagenic factors in selection was strongly influenced at its first stage by the practical aspect. While solving directly the problem of breeding new, highly potent microorganisms producing varicus substances the scientists concentrated their attention on the practical results of using mutagen-induced variation rather than on studying the peculiarities of the process. The main result of this first stage of work was the proof of a great effectiveness of mutagenic factors for the breeding of productive forms, as compared with the effectiveness of selection based on spontaneous (natural) variation.

On the other hand the same practical purposes made it necessary to solve a number of principal problems of induced variation in microorganisms. Practical selection began to put forward special problems of theory, due to the fact that at a certain stage the rate of selection with the use of mutagenic factors in highly active strains, as we have already mentioned above, began to fall down and it appeared to be necessary to find out the causes of this phenomenon and the ways to overcome it.

On the other hand it seemed to be very important to learn how to control the mutation process, i.e., how to obtain the desired variation. The problem consisted of finding out the ways for preferential obtaining of desired variation.

Thus, while estimating the results of the 15-year period of using

microbial mutations for industrial purposes one may subdivide the whole course of this work into two stages: The first stage is characterized by maximal use of mutagens for breeding new, more productive strains of industrial microorganisms. The second stage is characterized by the expansion of research work on the regularities of induced variation in microorganisms with respect to their quantitative features that play a very important role in industry.

Among the works illustrating the first stage one should mention the experience of breeding the Wisconsin line of *Penicillium chrysogenum* strains (Backus and Stauffer, 1955; Stauffer, 1961), the results of the selection of streptomycin-producing strain described by Dulaney (1953), the genealogy of penicillin-producing strains (Alikhanian, 1956; Alikhanian *et al.*, 1956; Alikhanian and Mindlin, 1956), and the results of the selection of strains producing tetracyclines (Alikhanian *et al.*, 1959a) (see Figs. 1 and 2).

Among the strains of the Wisconsin series special attention should be paid to strains Q-176, BL3-D10(pigmentless), Wis 49-133, and Wis 51-20.[1] These strains have played a very important role in the sharp increase of penicillin production in many countries of the world. The productivity of these strains, as reported by Backus and Stauffer, reached 2500 units/ml.

The strains mentioned, as well as our strains "New Sort," G-31, and "New Hybrid," highly potent streptomycin-producing strains selected by Dulaney, highly potent strains producing chloro- and oxytetracycline (Alikhanian *et al.*, 1959a) have given one sufficient reason to believe that the use of physical and chemical factors signifies a new era in selection, in general, and in microbial selection, in particular. This stage seems to us to be a great event in the history of selection for it has shown that man is able to increase the productivity of living beings many dozens or even hundreds of times within comparatively short periods of time.

The ideas of selection formed for decades or even centuries are now acquiring new content which demands its thorough studying.

The principal properties of microorganisms that were of interest for selectionists at all stages of the development of this science consisted chiefly of features which might be estimated by quantitative

[1] In this as well as in all other cases we proceed from the figures of antibiotic production obtained in similar conditions of fermentation and nutrient medium, i.e., in conditions comparable for various strains.

methods, or—with a very rare exception—of alternative features which were also of importance in the production process.

At the very outset of radiogenetics and radioselection Baur (1925) and Stubbe (1929) suggested that the frequency of small and weak hereditary changes which are of great importance for selecting economically valuable features are actually much higher than that which we are able to discover. The whole complexity of

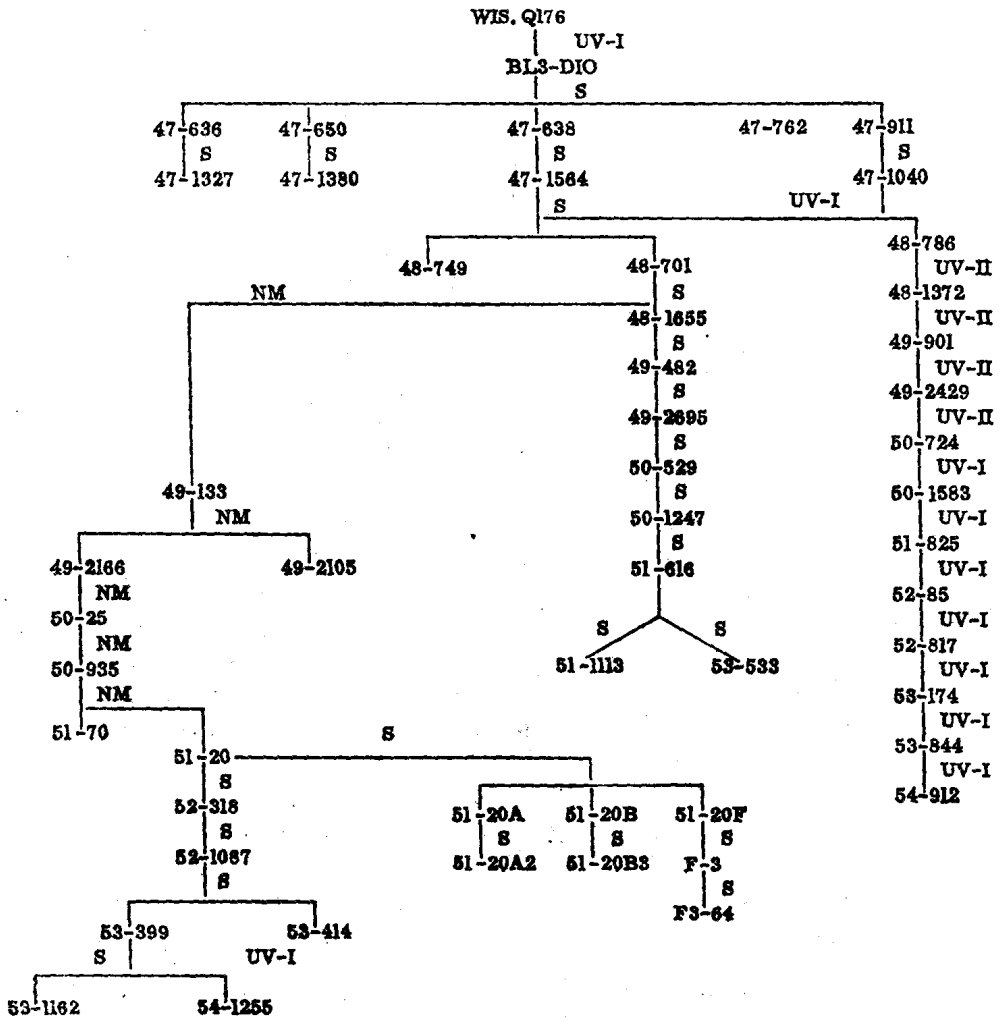

FIG. 1. Genealogy of the Wisconsin penicillin-producing strains. (After Stauffer, 1961.)

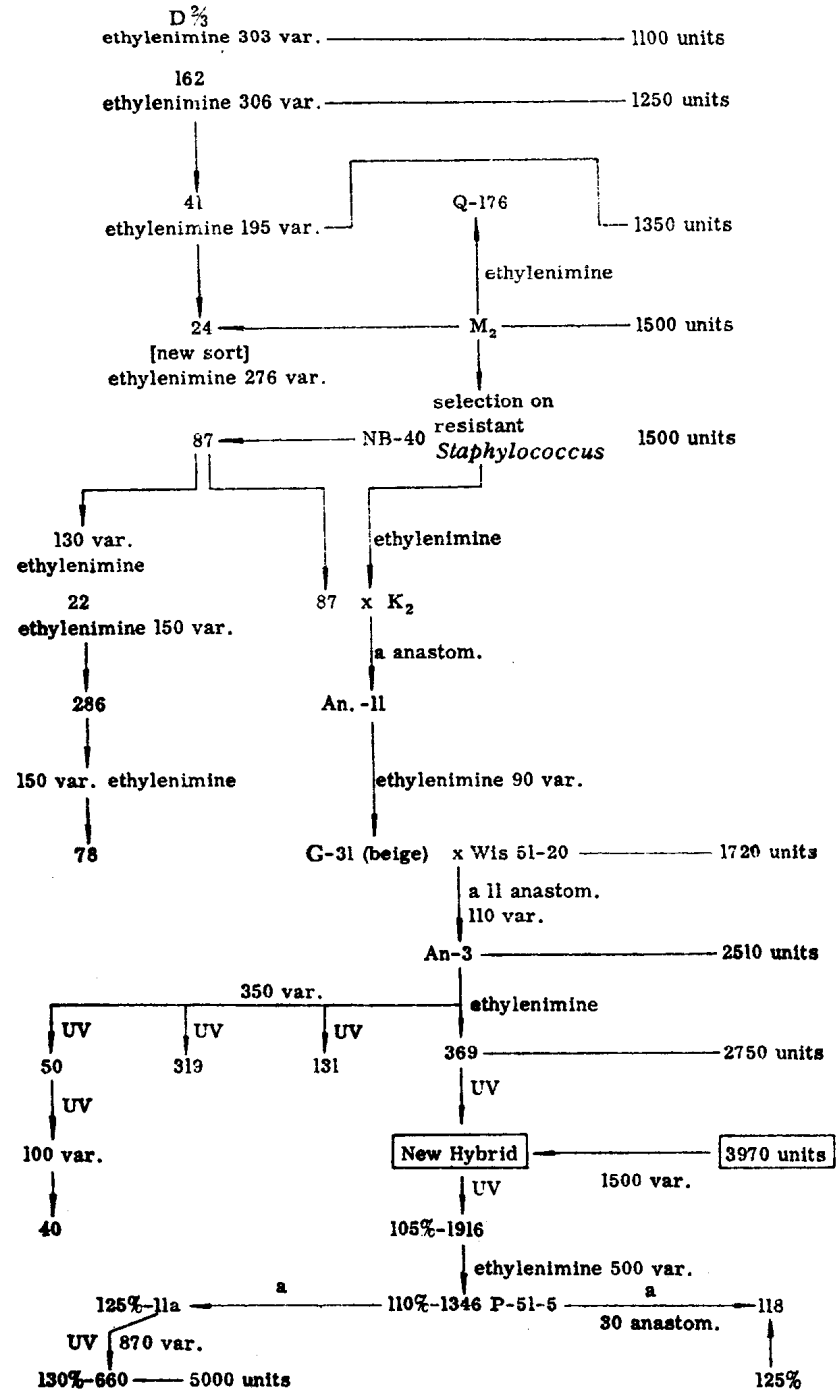

FIG. 2. Genealogy of the Soviet penicillin-producing strains. (After Alikhanian *et al.*, 1959a.)

the problem of enhancing quantitative features consists in the necessity of developing a reliable system for the detection of these features and their fixation in the genotype.

This is why we consider it very important for radioselection of microorganisms to solve the problem pertaining to the effect of selection and to the development of methods for fixation of all hereditary changes of quantitative features induced by various mutagens.

If the newly appearing changes in diploid organisms is connected with crossing, with further analysis in generations, and with "homozygoting" of recessive mutations, then the main difficulty in detecting changes in microorganisms is connected with the peculiarities of biosynthesis of various compounds, which are the main features to be selected.

The development of radioselection was especially complicated by the fact that after the discovery of the mutagenic force of X-rays and then of other kinds of radiation and chemical compounds, all investigations were based on studying visible and/or lethal mutations. Visible and lethal mutations were unmistakably determined and therefore comparatively easily calculated and were taken as a basis for revelation of genetic regularities. Mutations connected with the changes of quantitative features, were, as a rule, overlooked.

Thus, besides the necessity of obtaining practically valuable results by using mutagens in microorganisms, science had another mission, namely, studying the regularities of induced microbial variation with respect to quantitative features. Both aspects required the solution of a number of problems, many of which have not yet been solved.

II. Role of Major Mutation

The use of mutagenic factors in the work with microorganisms for industrial purposes has two aspects. The first aspect concerns the obtaining of major mutations changing pronouncedly a feature which is of practical value. One can give a number of examples concerning the improvement of a production process due to the employment of such mutations.

As is known, all penicillin-producing strains of *P. chrysogenum* into the nutrient medium gave a golden-yellow pigment which reduced the yields of the antibiotic at the stage of isolation.

By treating these strains with UV-rays it is comparatively easy to obtain pigmentless mutations. Such mutations have been obtained, although, as soon as a pigmentless mutation sprang up, the productivity of the strain fell down by 25–30 % (Alikhanian and Borisova, 1961). A fall in the productivity was later observed with other morphological mutations in microorganisms, producing both penicillin and other antibiotics. Oleandomycin-producing microorganism *Actinomyces antibioticus* excretes a dark, almost black pigment into the medium. When treated with three mutagenic factors (UV- and X-rays as well as ethylenimine) it gives many pigmentless mutations. In all cases the productivity of these strains was much lower than that of their pigment-producing parents, although in this, as well as in the case with penicillin producer, there was no connection between the mechanism of antibiotic production and the pigment excretion. In the case with oleandomycin producer the loss of productivity in pigmentless strains may be compensated or even overlapped by selection whereas the loss of pigment excretion may be of value for the process of the antibiotic isolation.

A similar case of a "major" mutation is described in a streptomycin-producing strain. The initial strain produced up to 45% of mannosidostreptomycin. Of a great number of single-spore UV- and X-ray variants tested a mutation was selected which sharply changed the ratio between streptomycin and mannosidostreptomycin. The amount of mannosidostreptomycin produced by the new mutant reached 5%.

Another example of "major" mutations in antibiotic-producing microorganisms is a mutant strain of *Actinomyces rimosus* (*Streptomyces rimosus*), the oxytetracycline producer, obtained as a result of exposing its parent to UV-irradiation. Contrary to many other strains that reduced their antibiotic production sharply with a concentration of inorganic phosphorus in the medium over 4–5 mg./100 ml. the mutant strain in question, i.e., strain *A. rimosus* LS-T-293, produced the maximal amount of the antibiotic with a concentration of inorganic phosphorus in the medium of 8–9 mg./100 ml. (Alikhanian *et al.*, 1959b, 1961b).

In recent years the use of major mutations in microorganisms has acquired particular significance and has even taken the form of a new direction in their selection.

It was shown that the treatment with mutagenic factors may give rise to mutant strains synthesizing antibiotics with a changed chemi-

cal structure. In some cases, e.g., in the case described by a group of American authors, a comparatively insignificant change in the chemical structure of an antibiotic (it was chlorotetracycline in this particular case) almost completely deprives the latter of its antibacterial properties. This phenomenon was observed, in particular, after dehydrogenation at C-5a in the molecule of chlorotetracycline, as well as after some other changes in the chemical structure of this antibiotic (McCormick *et al.*, 1957, 1958a, b). On the contrary, in other cases a change in the chemical structure of an antibiotic resulted in obtaining substances with a rather high antibacterial activity but with an altered antimicrobial spectrum (Ballio *et al.*, 1960). Both cases, as it will be shown below, are of great practical importance.

McCormick *et al.* (1960) and Alikhanian *et al.* (1961d) have shown almost simultaneously that mutual cultivation of two mutants of *Actinomyces* (*Streptomyces*) *aureofaciens* (McCormick) and *A. rimosus* (Alikhanian) with disturbed biosynthesis of the antibiotics yields a great amount of the active product. In both cases the authors assumed that the synthesis of the antibiotics by these mutants is blocked at its different stages. But when such mutants are cultivated together they become mutually complementary and the synthesis of the normal product is restored. On the basis of these studies a group of American investigators succeeded in isolating a specific substance from the fermentation broth of an inactive mutant of *A. aureofaciens*. This substance had catalytic properties and promoted conversion of 7-chloro-5a(11α)-dihydrotetracycline produced by another inactive mutant into 7-chlorotetracycline, probably being a precursor of the latter (McCormick *et al.*, 1958b). This substance was named Co-synthetic factor I (Miller *et al.*, 1960).

A substance with catalytic properties but different from co-factor I and therefore named factor X was isolated from the fermentation broth of an inactive mutant of *A. rimosus* (Alikhanian *et al.*, 1961b; Zaitzeva *et al.*, 1961). It is evident from the above that the use of mutants with disturbed antibiotic production is a very important way for studying and deciphering the mode of antibiotic biosynthesis and may contribute to the isolation and identification of their precursors. Indeed, as a result of studying UV-mutants of *Nocardia rugosa* with altered biosynthesis a number of rather interesting data on the specific precursors of the pseudo-porphyrin ring of

vitamin B_{12} were obtained. These data contributed to the determination of the sequence of chemical reactions leading to the biosynthesis of this vitamin (Barchielli *et al.*, 1960; Di Marco *et al.*, 1961).

Ballio, Chain, and others studied changes in the antibacterial spectrum of penicillin that take place as a result of incorporation of α, ω-dicarbonic acids into its side chain. They succeeded in isolating a variant of strain *P. chrysogenum* Wis 51-20. On addition of adipic acid to the fermentation medium this variant synthesized (4-carboxy-*n*-butyl)penicillin, a new type of penicillin. Contrary to benzylpenicillin this type of penicillin inhibited chiefly gram-negative microbes and was very close in its action to cephalosporin N (Ballio *et al.*, 1960).

A program of selecting mutant strains of *P. chrysogenum* with an altered antibacterial spectrum is outlined by Sermonti and Morpurgo (1957). Using a test organism almost not affected by the antibiotic produced by the initial strain the authors succeeded in selecting variants producing an antibiotic with a preferential activity against gram-negative bacteria (*Klebsiella pneumoniae, Bacillus cereus*).

The cases with oxy- and chlorotetracycline-producing strains described by us show how successfully one may use mutations not only for studying the mode of biosynthesis but for obtaining microorganisms synthesizing valuable chemical compounds, although generally similar to the primary forms, but nevertheless different from each other and having the most unexpected biological, pharmacological, and therapeutic properties.

The prospects of employing microbial mutations, especially for obtaining substances with the most unexpected structures seem to us rather promising. The most effective way, in our opinion, is the employment of mutants for breeding organisms producing new antibiotics active against those groups of microbes which are still resistant to antibiotics produced by microorganisms found in natural populations. We think it rather tempting to use for this purpose highly productive strains of microorganisms producing broad-spectrum antibiotics. As is known some mutants produce chemical structures. An insignificant reconstruction of the molecule of an antibiotic by mutant strain may lead to new biological, pharmacological, and therapeutic properties of a known antibiotic.

Of great interest in this connection is a paper by Kelner (1949).

In 1949 Kelner selected 7 actinomycete cultures (out of 15) which either did not inhibit *Micrococcus lysodeikticus*, *S. aureus*, and *E. coli* at all, or showed very small inhibition zones (0–2 mm. in diameter). Two of 7 cultures inhibited the growth of one test organism but did not affect the other.

As the mutagen UV- and X-rays were employed. The dose of the latter was 300,000 rentgen units. Control cultures were not irradiated.

Having examined several thousand irradiated cultures Kelner found that each parent form contained mutants showing zones of inhibition of the test microbes. This experiment may be illustrated by a table taken from Kelner's paper (see Table I).

The next table (Table II) shows data concerning the antibacterial spectrum of several antibiotically active mutants and their comparison with the parent forms. As it is evident from this table, one and the same culture treated with mutagenic factors may yield several forms differing from each other and producing different antibiotics.

For example, among mutants isolated from *S. flaveolus* there are forms producing at least three qualitatively different antibiotics. Among mutants isolated from *S. griseus* there are forms producing at least three and possibly four antibiotics.

Thus, it is quite possible to assume that irradiation of spores of an antibiotically negative culture may give active forms. The fact that it is the mutagen that induces the active forms is proved by the experiments with a nontreated culture where the percentage of active variants is much lower.

The difference in the antibacterial spectra of the mutants shows that in some cases qualitative changes in the chemical composition of the antibiotic take place. With terramycin and aureomycin this may now be taken for granted. Thus it is possible to assume that induced mutations may serve us as a means for creating numerous chemically different antibiotics.

It is quite possible to agree with Kelner that any microorganism in the course of its growth and autolysis must produce very small quantities of thousands of metabolic products most of which are quite unknown as to their nature and biological activity.

Mutants of many microorganisms may produce these substances in quantities sufficient for their discovery, at least, by biological methods. Therefore, we have means for obtaining sufficient quanti-

TABLE I

PRODUCTION AND FREQUENCY OF ANTIBIOTICALLY ACTIVE MUTANTS IN IRRADIATED ACTINOMYCETE SUSPENSIONS

Actinomycete	Irradiation	Medium	*Micrococcus lysodeikticus*		*Staphylococcus aureus*		*Escherichia coli*	
			No. colonies tested	% Mutants	No. colonies tested	% Mutants	No. colonies tested	% Mutants
S. *albosporeus* ATC 3003	X-ray	Nutrient	—	—	—	—	6630	0.03
		Yeast extract	—	—	—	—	6900	0.04
S. *albus* ATC 3004	X-ray	Starch tryptone	710	0.0	—	—	710	0.0
S. *cellulosae* ATC 3313	X-ray	Nutrient	—	—	—	—	9350	—[a]
		Yeast extract	—	—	—	—	7400	—[a]
S. *flaveolus* ATC 3319	X-ray	Nutrient	3280	0.48	—	—	2580	0.0
		Yeast extract	5200	0.75	—	—	3100	0.0
		Starch tryptone	3800	1.9	—	—	3400	0.0
		Glucose nutrient	10,700	0.44	—	—	10,500	0.0
	UV-light	Yeast extract	10,890	0.03	—	—	—	—
	None	Yeast extract	28,180	0.004	—	—	16,400	—
S. *griseus* ATC 3326	X-ray	Nutrient	—	—	—	—	16,400	—[b]
		Yeast extract	—	—	—	—	8240	0.03
		Starch tryptone	1900	0.4	—	—	12,400	0.02
	UV-light	Nutrient	—	—	—	—	21,520	0.02
		Nutrient	—	—	—	—	8640	0.0[c]
S. *violaceus* ATC 3355	X-ray	Nutrient	7020	0.06	—	—	7000	0.0
		Yeast extract	3360	0.03	—	—	3790	0.0
S. *viridochromogenes* ATC 3356	X-ray	Nutrient	—	—	2420	1.0	2600	0.04
	None	Nutrient	—	—	18,950	0.02	—	—

[a] Presence of productive mutants questionable.

[b] Mutants present but number not ascertained.

[c] Spores irradiated after being spread over agar plate.