MUTATION OF PHYTOMONAS STEWARTII BY X-RAY IRRADIATION[1]*

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VARIATION in cultural characteristics, viability, and motility of bacteria has been recognized almost from the beginning of their study in pure culture. Because of the influence of the Koch school, which emphasized cultural purity, early bacteriologists were prone to attribute changes in cultures to contamination. Thus, many of the earlier observations of bacterial variation probably were discounted or misinterpreted. Variations associated with virulence and antigenic types, however, have become so important, particularly from the medical standpoint, that in certain forms such as the pneumococci many variant types have been described, and many of the cultural conditions necessary for the full growth of the particular variant types have been made known. Lacking critical experiments to prove their cause and nature, these variant strains ordinarily have been referred to as dissociants, variants, and saltants arising by some process vaguely defined as "dissociation," and, with the exception of certain workers, the terms "mutant" and "mutation" as ordinarily applied to variation in higher organisms such as maize, Drosophila, and man have been avoided.

Superficially, if we neglect the differences in reproductive capacity of the mutants, the observed changes and the experimental conditions surrounding the changes suggest that variation is caused by the environment acting on the organism rather than by spontaneous changes within the organism. In a proper environment a parent culture may be replaced by one of its variants. In some cases the environment necessary to facilitate the appearance of these variants is known and may be controlled to some degree. In light of such facts it is not surprising that environmental differences have been interpreted as causative in initiating variation rather than correlative and that the mutation concept is often regarded as unnecessary to account for the variants observed.

The problem of the origin of variation is of particular importance to the bacteriologists' concepts of species and the geneticists' outlook on the mech-

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anism and permanence of inheritance. To analyze this problem, we need to know the quantitative aspects of the behavior of the parent and mutant bacterial forms under a variety of environments. Then an analogy may be drawn to the same quantitative reaction as is expressed by specific genes under like conditions in higher forms where mutation is the mechanism considered responsible for variation. Penetrating irradiation offers one means of studying this problem, the success of which has been demonstrated in analyzing the fundamental aspects of variation and heredity in multicellular organisms.

Little use has been made of irradiation in modifying the hereditary constitution of the bacterial cell. Experiments with X-rays on unicellular organims have dealt largely with inactivation or killing effects rather than with mutation. Either the mutations observed were few or they were regarded as quantitatively unimportant. Those that have been reported have dealt with immunological and growth phenomena, as in the report of LANGE and FRAENKEL (1923) on the reduced virulence of a human strain of tubercle bacillus when inoculated into guinea pigs, or have been qualitative, as in the report of Haberman and Ellsworth (1940) in which they show that X-rays increased the amount of "dissociation" in *Staphylococcus aureus* and *Serratia marcescens*.

This paper presents a study of variation in the unicellular organism *Phytomonas stewartii* (E.F.S.) Bergey, et al., as it occurs spontaneously on culture media and as it occurs on the same media after the organism has been exposed to X-ray irradiation. *Ph. stewartii* is a non-motile bacterium of medium length. Its natural host is maize, in which it produces a vascular wilt disease. The organism may assume epiphytotic virulence for maize in certain areas when local conditions have been favorable. On solid media each bacterial strain has a definite growth configuration as expressed in colony type and size. Colony color is normally yellow. A quantitative estimate of the type and rate of occurrence of hereditary variation in these characters both spontaneously and during exposure to X-ray irradiation is presented below.

METHODS

Bacteria were irradiated with X-rays from a gas-type tube of the general design of WYCKOFF and LAGSDIN (1930). An exact description of this particular tube has been presented by PINNEY (1939). The targets were pure metals—copper or silver. The majority of the experiments were with X-rays from the copper target. The X-rays were filtered heavily by a window of palladium for the silver target and nickel for the copper target, the thickness of the window being adjusted to absorb about 50 percent of the irradiation. The current through the tube during irradiation was held constant at 12.5 milli-amperes. Beam intensity at the time of irradiation was

measured either by a small ionization chamber or a Victoreen dosimeter. The ionization chamber was designed by PINNEY following the general plan of TAYLOR and SINGER (1930). The Victoreen dosimeter was checked frequently with this ionization chamber.[2]

The average effective wave length of the X-rays obtained from irradiation of the silver and copper targets under the above conditions was determined by absorption experiments through successive sheets of aluminum 13×10^{-4} and 72×10^{-4} cm in thickness. Results of these tests showed the average effective wave length of the silver irradiations to be 0.7Å (17.6 kv) and of the copper to be 1.5Å (8.2 kv).

Bacteria were irradiated in straight-walled dishes 18 mm in diameter and 5 mm deep made from a block of paraffin. Into each dish 0.75 ml of 14–18 hour nutrient broth culture of *Ph. stewartii* was introduced, and the dish was sealed with cellophane to prevent air contamination. The number of living bacterial cells after treatment was estimated from the known inactivation rate established under like conditions in previous experiments. Employing this estimate, a dilution was made that allowed most colonies on a plate to develop individually. The number of colonies desired varied with the characteristic size and consistency of the colony of the strain treated. One drop of broth of the desired dilution was placed on the surface of the hardened nutrient dextrose agar plate and smeared evenly over the entire surface of the plate by means of an L-shaped glass rod. Plates were incubated at room temperatures for 48 hours, then observed at 16× magnification for colony changes from the normal as regards color, surface, or size. The observed variants were suspended in broth and plated again to ascertain whether or not the variations were genetic. Amount and kind of variation present before treatment was determined in all cases. All dilutions and smearing of the bacteria on the plates were completed in less than 20 minutes after treatment. Since the maximum time of irradiation was 25 minutes, treatment and plating was completed in a period much less than the generation time of *Ph. stewartii*, which is greater than 1.5 hours in the media used.

Since the bacteria were suspended in broth, special techniques were neccessary to measure the X-ray dosage. Since these problems will be discussed in another paper, we shall limit the data herein to the following: the frequency of variants under natural conditions, the changes in this frequency brought about by irradiation with X-rays, a description of the frequency and rates at which the different types of variants appear under the circumstances, and the virulence and stability of the different variants.

Experiments with the effect of X-rays of different wave lengths on the

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inactivation of these bacteria showed that survival during irradiation follows the simple exponential function in which the survival is inversely proportional to the dosage. The general equation for this relationship is

$$\text{survival ratio} = e^{-ar}$$

where a is a constant and r is the roentgens to which the bacteria are exposed. The fact that inactivation follows this expression suggests that one unit of energy absorbed by the bacterial cell is sufficient to kill it if the absorption takes place in the vital region. This expression holds under a variety of conditions and thus may be used as an accurate measure of roentgen energy absorbed by the bacteria under conditions of treatment where it is difficult to measure the absorbed energy directly. For this purpose the expression above takes the following form:

$$r = \frac{\log \text{survival}}{a \log e}.$$

Since a and log e are constants, the log of the survival ratio is a sufficiently accurate measure of dosage. This measure is the one used in the text.

Throughout this work it was assumed that most colonies start from a single bacterium and that any change in the heredity of the individual cell that affects colony color, type, or size may be observed after the cell is placed on agar and allowed to develop into a colony. The assumption as to the single-cell origin of these colonies is not appreciably "less real than ideal" DUGGAR (1936) and has experimental verification in the work of MCNEW (1938) and LINCOLN and GOWEN (in press). By direct observation of seeded poured plates MCNEW observed that cells of *Ph. stewartii* were distributed singly throughout the agar in all but about 1 percent of the cases. LINCOLN and GOWEN, by studying the progeny of mixtures of yellow and white or rough and smooth strains of *Ph. stewartii*, showed that the bacteria in these mixed cultures had an equally low rate of sticking together. Furthermore, X-ray experiments on this species showed that the bacteria were inactivated in a manner comparable to individual particles as contrasted to the curve characteristic of two or more attached particles. The observation that, except for the sectored colony types, most mutant colonies are pure and stable from the time of origin is a further indication of the single-cell origin of most colonies.

BACTERIAL STRAINS USED AND KIND OF VARIATION OBSERVED

Two strains of *Ph. stewartii* were used for these experiments—S15 (400), a rough colony type, and A14 (500), a smooth colony type.

Classified in table 1 is the variation observed in S15 stock. The normal colony type of S15 (Plate I, A) is a small, compact, slightly rough, dark

yellow colony. This type has arbitrarily been called RI and other rough types designated by RII—RVI (Plate I, B, C, D, E). These types differ principally in the degree of roughness and in the elevation of the colony. Smooth variants were observed, but no attempt was made to classify these into a smaller unit, because any particular smooth variant occurred less frequently than did any particular rough variant. Smooth types varied

TABLE 1

Occurrence of mutation in Phytomonas stewartii under natural conditions and after X-ray treatment.

CHARACTERS OBSERVED STOCK S15 (400)	NUMBER OF MUTANTS OBSERVED		
PARENT TYPE IS DARK YELLOW IN COLOR, ROUGH I IN COLONY TYPE	SPONTANEOUS (BROTH)*	AFTER IRRADIATION	
		17.6 kv	8.2 kv
Mutational changes involving only one character			
Color change from dark yellow to:			
Pale yellow	29	11	72
White	16	3	22
Colony type change from Rough I to:			
Rough II	8	14	38
Rough III	2	5	4
Rough IV	2	2	4
Rough V	0	1	3
Rough VI	0	0	1
Smooth type	16	4	4
Colony size change from normal to:			
Small	1	18	10
Mutational changes involving more than one character			
Pale yellow—Rough II	1	2	2
Pale yellow—Rough III	0	1	1
Pale yellow—Rough IV	0	4	6
White—Rough II	1	0	12
White—Smooth	0	1	0
Yellow—Smooth—Mucoid	2	0	1
Small colony—Pale yellow—Rough I	0	6	2
Small colony—Pale yellow—Rough II	1	14	5
Small colony—Pale yellow—Rough III	0	2	1
Small colony—White—Rough I	1	5	1
Unstable variants	0	11	4
Sectors	12	36	80
Total	92	140	272
Colonies observed	1,524,000	9,360	386,000

* Minimum number. See text.

from homogeneously granular to translucent (Plate I, F, G, H, I, J). The predominant smooth type was an intermediate form developing a chromogenic center within a translucent outer portion. The pattern of the chromogenic material was relatively constant in a given environment but between environments was subject to change more than the other characters described. Mucoid smooth forms from the non-mucoid S15 rough type were observed. Small colony types (Plate I, K) occurred, particularly after irradiation. In stock S15 the change of colony color from dark yellow to pale yellow or to white represented another frequent change. Sectored colonies of the various types also were observed (Plate I, L, M, N), as were other colonies classified as unstable because stocks of such types could not be purified for the variant characteristics. The change of colony color from dark yellow to pale yellow and to white was observed.

Strain A14 is characterized by large, spreading, smooth, mucoid diffusely grayish-colored colonies (Plate II, A). Variation within this strain has been studied less than in the S15 strain because the colonies of A14 are less favorable material for variation study than are those of S15 due to their tendency to coalesce and their larger size.

The variations observed in A14 are classified in table 2. Two groups of smooth variants were observed frequently. The first group (Plate II, B, C, D, E, F) was similar to the parent in size and mucoidness of colony, but the second group (Plate II, G, H, I, J, K, L) was composed of dry mucoid, small-colonied smooth types consisting entirely of highly chromogenic material. In this latter group a single mutation from mucoid to non-mucoid was observed. A rough-mucoid type (Plate II, K) appeared to be the mutant commonly observed to occur during susceptible host passage (LINCOLN 1940) and appeared completely rough for the first two days' growth, then formed a smooth halo around the rough center. Completely rough mutants of two types were observed (Plate II, M, N). A rather frequently ob-

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PLATE I. Mutants observed in the S15 (400) series.

A.—Parent type. Colony highly chromogenic, dark yellow in color, and of RI type surface appearance.

B, C, D, E.—Other rough types of colonies (RII, RIII, RIV, and RV, respectively) observed as mutations from A. All are composed entirely of chromogenic material.

F, G, H, I, J.—Smooth types of colonies observed as mutations from A. F is entirely chromogenic, while J is entirely non-chromogenic. G, H, and I are intermediate forms having a center of chromogenic material surrounded by non-chromogenic growth.

K.—Small colonies in which are mixed two normal sized colonies to show comparative size. ×1½. Four day old colonies.

L.—Sector. RIV sector arising in RI colony. ×5.

M and N.—Sectors. Bursts typical of growths occurring from the unstable type of colonies. ×2. Three day old colonies.

Colonies A–J magnified 4 diameters and photographed by reflected light after 48 hours' growth on nutrient dextrose agar.

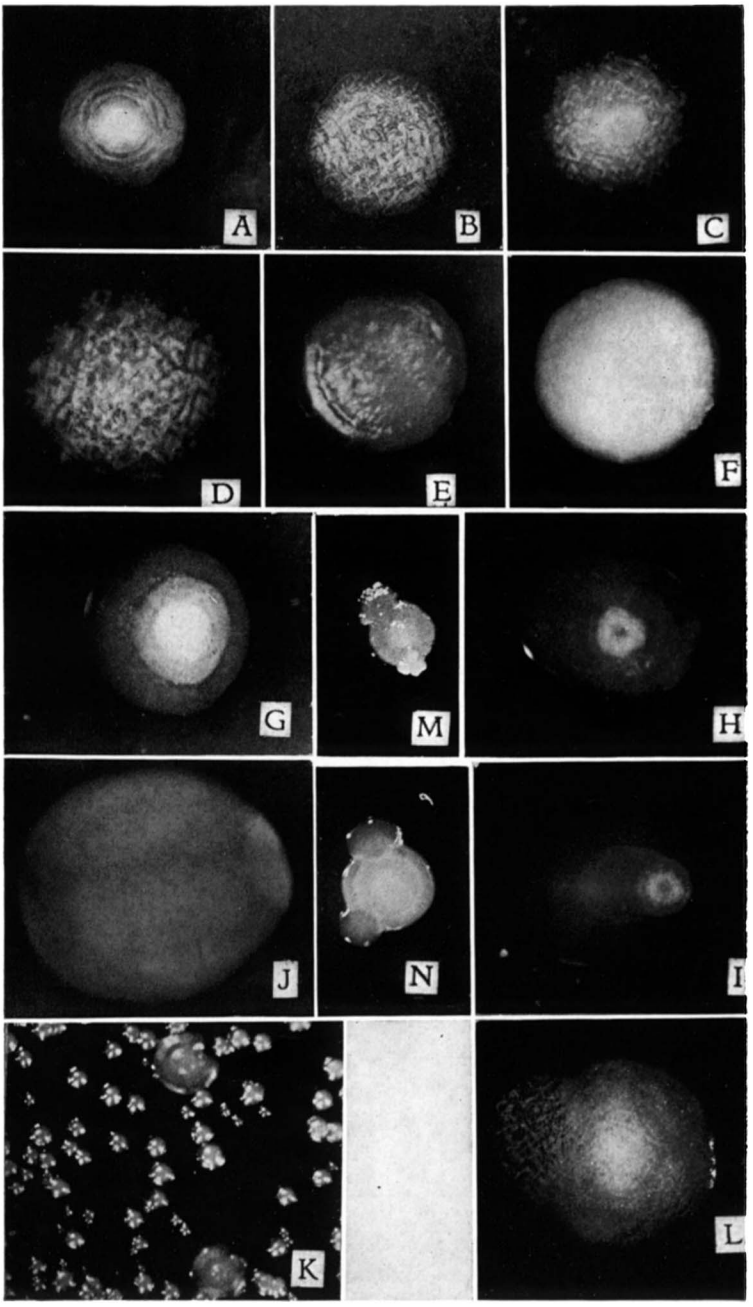

PLATE I

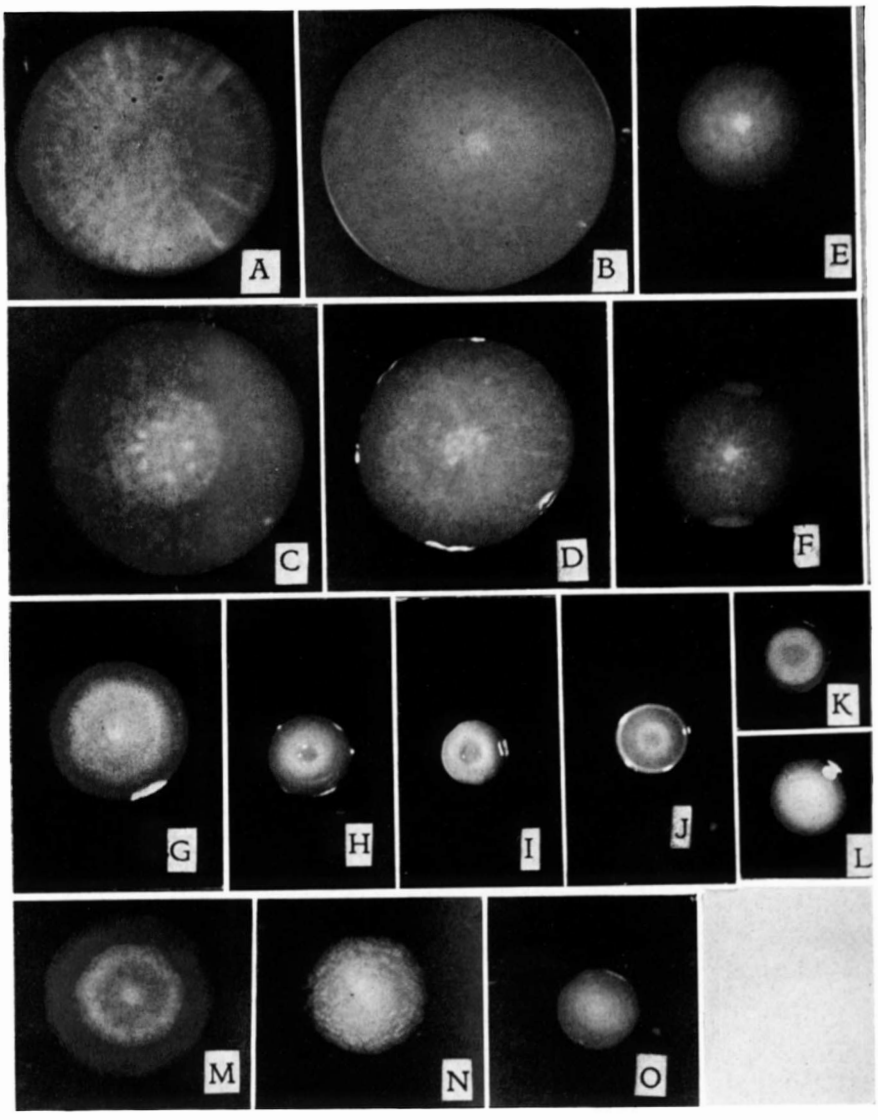

PLATE II

TABLE 2

Occurrence of mutation in Phytomonas stewartii under natural conditions and after X-ray treatment. (17.6 kv).

CHARACTERS OBSERVED STOCK A14 (500)	NUMBER OF MUTANTS OBSERVED	
NORMAL IS PALE YELLOW COLOR, SMOOTH COLONY WITH SLIGHTLY STRIATED CENTER, VERY MUCOID.	SPONTANEOUS (BROTH)*	AFTER IRRADIATION 17.6 KV
Mutational changes involving only one character.		
Color change from pale yellow to:		
Dark yellow	1	2
White	—	1
Colony type change from large smooth colony with undifferentiated center to:		
Large smooth, differentiated center	3	3
Small smooth, compact and dry	8	9
Rough center, mucoid smooth edge	2	1
Rough	—	2
Colony size change from normal to a small watery scant type of growth	4	3
Mutational change involving more than one character, from parental to:		
Dark yellow—Rough mucoid	—	1
White—Small dry smooth	—	1
Small smooth—Non-mucoid smooth	—	1
Unstable variant	—	1
Total	18	25
Colonies observed	156,000	24,378

* Minimum number. See text.

PLATE II. Mutants observed in the A14 (500) series.

A.—Parent type. Sticky-mucoid, large-colony type having slight internal striations of highly chromogenic material.

B, C, D, E, F.—Sticky-mucoid, large-colony, smooth types having a center differentiation of highly chromogenic material.

G, H, I, J, K, L.—Dry-mucoid, small-colony, smooth types consisting entirely of highly chromogenic material.

M.—Intermediate (RM) type. Center chromogenic material is rough type of dry-mucoid consistency (like G to O); outer, halo-like material is non-chromogenic and is of sticky-mucoid consistency (like A to F).

N and O.—Rough types consisting entirely of highly chromogenic material of dry-mucoid consistency.

Colonies of figures C, F, and G were observed only from spontaneous variation; other figures were observed after X-ray treatment. Photographed by reflected light. Colonies 48-hours old on nutrient dextrose agar. Magnification ×3.

served small, translucent colony type proved to be the only mutant difficult to maintain on culture media. In addition to these morphological mutations the changes from pale yellow to dark yellow and to white were observed.

RATE OF MUTATION OF COLONY CHARACTERISTICS

Summarized in table 1 are the data from numerous experiments to determine the spontaneous mutation rate of colony color, surface, and size characteristics. For this material, bacteria from a single colony were used to inoculate a tube of nutrient broth. After 18–20 hours' growth at 27°C the cultures were diluted, surface-plated on agar, and grown for 48 hours. Colonies then were examined for variants, and a minimum mutation rate was calculated based on the tube as a unit. Repeated observation of the same variant in a single tube was considered to indicate merely its growth after origin and not its repeated origin. In these experiments the minimum mutation rate ranged from zero to 137 mutants per million cells observed, the average being 46.5. Most uncontrolled factors, such as mutant cell lethality, lower viability, a possible segregation mechanism, and random loss of mutants in sampling, tend to keep the observed mutation rate at a minimum. The average and the range of the minimum mutation rates were of the same order of magnitude as those observed for mutation of specific characters in higher organisms where observed variants are ordinarily attributed to mutation of specific genes.

The different types of mutation with S15 stock are separated in table 1 into three groups—those arising spontaneously and those treated with X-rays of 17.6 kv and 8.2 kv. X-ray mutants are not different qualitatively from those that appear spontaneously, since most types of mutations appear in each group. The difference in the occurrence of mutation under natural conditions and X-irradiation lies in the frequency of mutations rather than kind of mutation produced. An example of this fact is found in table 1. Spontaneous mutations were found in 92 bacteria out of 1,524,000 colonies observed. Of these 92 bacteria several varied in two or more characteristics making a total mutation of 109 different characters. For the bacteria treated with 8.2 kv there were 272 mutated bacteria out of 385,919. Two or more mutations occurred in several individuals of this group, the total being 310 mutations. The surviving cells observed for the different experiments from which these data were drawn ranged from 252 to 37,000,000 per 100,000,000 in the initial culture. If we choose the point where 8,000 organisms survived out of the initial 100,000,000, the rate of mutational change was 4.2 per 1,000 colonies. This point corresponds to roughly 38,000 roentgens incident to the bacteria. The average (and distinctly tentative) rate of visible mutation per roentgen per bacteria there-

fore corresponds to 1.1×10^{-7}. As may be noted from table 1, three major characteristics were emphasized in determining the frequency of these different changes. Colony color may be dark yellow, pale yellow, or white. Colony surface may be smooth or rough, the degree of surface roughness being dependent upon the particular mutational change. At least five different classifiable and subsequently reproducible mutations affecting surface roughness are found. Colony size may vary from a very small colony to one of two or three times the size of the parent type. The average mutation rate per locus studied therefore would be less than 1.1×10^{-7} by at least a factor of three to account for the three different characters utilized in determining these frequencies. In this paper the rate of change of these bacteria under X-rays will be considered as approximately 3.7×10^{-8} per character per roentgen.

In their work on the effect of X-rays of 6.0 kv, 8.3 kv, and 17.4 kv, FRYER and GOWEN (1941) found 49 visible mutations in 168,945 gametes treated with roentgen dosages between 2,475 and 7,250 incident to the *Drosophila melanogaster* sperm. The rate of change per gene per roentgen was calculated as 7.0×10^{-8}. Besides these verifiable mutations following X-ray treatment, F_1 individuals were observed with one of the recessive characters. The occurrence of these individuals indicate either a mutation of the irradiated wild type allele to the recessive type or a deficiency in that region. Tests for the genic nature of these later changes could not be made because of sterility or early death of the mutant. The total of these changes plus the verified gene mutations was 123, or the rate of observed mutational change was 1.7×10^{-7} per gene per roentgen. This rate of change may be high as judged by the rates of mutation obtained by other workers. For the loci of forked, miniature, and eosin the data collected by TIMOFEEFF-RESSOVSKY (1933) indicate rates of 6.1×10^{-8}, 2.4×10^{-8}, and 2.6×10^{-8}. For sex chromosome genes MOORE, cited by JOHNSTON and WINCHESTER (1934), found 48 mutations in 116,200 gametes following a dosage of 3,975 roentgens or a mutational rate of 1.0×10^{-7}. The rates of mutation observed in this paper for *Ph. stewartii* are therefore easily within the range of those that one might expect for the genes of higher organisms.

One possible criticism against comparing the spontaneous and X-ray mutations should perhaps be discussed. Since the X-rays kill off or inactivate the bacteria from 100,000,000 down to 1,000, it might be argued that the killing occurred chiefly in the parental form, thus leaving any spontaneous mutants to show a spurious increase in number. This criticism is easily put to experimental test where the inactivation rates of mutant and parental forms are determined for like roentgen dosages. We have made several such tests on mutants of this bacterium and have always found the same inactivation rates for the different mutants and their parents. This

furnishes strong evidence that we are dealing with true genes and gene mutations.

In the data of table 1 the distribution of the different types of mutations as they arose spontaneously contrasted with the same types as they arose through X-rays show a highly significant difference, the χ^2 value being 70 for 7 degrees of freedom. The percentage relative frequency of different mutations as reported in table 1 is shown in table 3.

TABLE 3

Percentage frequency of spontaneous mutations and mutations following X-ray treatment.

	SPONTANEOUS	X-RAY	P
Dark yellow to pale yellow	32	26	.15
Dark yellow to white	18	9	.01
Rough I to other roughs	15	24	.05
Rough I to smooth	18	2	.01
Normal colony size to small	3	13	.01
Stable to unstable forms	0	3	.01
Uniform colonies to sectored	12	23	.01
Normal to mucoid	2	1	.02

The mutation from dark yellow to pale yellow (table 3) occurs with nearly equal frequency in the spontaneous and in the X-ray group. However, 18 percent of the spontaneous in contrast to nine percent of the X-ray mutations are from dark yellow to white colonies, a difference that is highly significant. Significant differences in the percentage frequency of the mutations in the spontaneous and in the X-ray group are found for all other mutations observed. The mutations from Rough I to smooth colony types and from normal to mucoid occur at greater frequency spontaneously than during X-ray treatment. In the other cases the mutations are more frequent in the X-ray group than in the spontaneous group. The fact that no unstable mutants were found in the 109 mutations of the spontaneous class whereas 15 were found in the X-ray group of 310 mutations is of interest in view of a similar comparison observed in Drosophila mutation. Variegated types in Drosophila are more frequent in X-ray material than in untreated material.

Necessary differences in technique could explain some of the observed difference between spontaneous and X-rayed bacteria. Through necessity the spontaneous types represent the accumulated changes over a period of 18 or more hours' growth. At any one of these cell divisions a mutation could occur. If reproducing at the same growth rate as the type form, the mutation would increase geometrically in numbers but remain in the same proportion to the type cells. If the mutation has a faster or a slower growth

rate or is unstable, reverting to type, etc., the proportion would not represent the original rate of change. Such special conditions could create bias toward the type form in the spontaneous data not found in the X-rayed material.

The question arises as to whether or not similar differences in mutation frequency exist in material treated by two types of X-rays. Data on this point are presented in table 4.

TABLE 4

Percentage frequency of mutations following treatment with X-rays of 8.2 kv and 17.6 kv.

	8.2 KV	17.6 KV	P
Dark yellow to pale yellow	29	21	.06
Dark yellow to white	11	5	.01
Rough I to other roughs	25	24	.81
Rough I to smooth	1.6	2.6	.48
Normal size colony to small	6	24	.01
Stable to unstable forms	1	6	.01
Uniform colonies to sectorial	26	19	.08
Normal to mucoid	1	0	.48

Most of the mutation frequencies found in the material irradiated by X-rays of 8.2 kv are similar to those found when X-rays of 17.6 kv were used. There are three exceptions—namely, X-rays having the greater energy induced changes to the small colony type and to unstable forms with a greater frequency than the X-rays of the lower energy, while the change from dark yellow to white colony color occurred with lowered frequency.

A similar comparison has been made between the spontaneous mutations and the X-ray mutations in the A14 stock as tabulated in table 2. The numbers of mutations observed in these experiments are scarcely sufficient to establish differences in rates. This is borne out by the fact that tests of significance for the spontaneous changes and X-ray changes show that the proportion of different types of mutants is essentially the same in each condition.

The results thus far discussed show that besides greatly increasing the frequency with which most mutations occur, X-rays also may tend to increase the proportion of certain types of variants. The significant differences between mutation under natural and X-ray conditions do not obscure the fact that there is a definite uniformity in mutation under the two conditions. This similarity supports the view that the mechanism by which the change is accomplished is similar in the two cases.

MULTIPLE MUTATIONS IN S15 STOCK

Colony color, surface appearance, and size are characteristics that have been observed to mutate. Each characteristic generally mutates singly, the occurrence of individuals showing two or more simultaneous changes being a rare event. In the spontaneous mutant class the total number of mutant colonies observed, after omitting the unstable and sectored groups, included 74 with a single mutation, five with two mutations, and one with three mutations—a total of 80 mutant colonies or a total of 87 character changes in 1,523,988 bacteria. If the production of two mutations in the same individual were independent, the probability of mutation of two or more characters would be obtained from the exponential equation

$$ne^{-m}\left(1 + m + \frac{m^2}{1.2} + \frac{m^3}{1.2.3} \cdots\right)$$

in which m is the mean mutation rate.

The rate of multiple mutation is much greater than is expected from this probability. Actually, five colonies with two mutations and one with three mutations were observed. Comparably high rates of multiple mutation have been observed in the X-ray classes. The frequency of these different types of mutations is found in table 5.

TABLE 5

Frequency of bacteria showing all parental type characteristics, one mutant character, two mutant characters, and three mutant characters.

VARIANT CHARACTER PER BACTERIUM	ACTUAL			EXPECTED*		
	SPONTANEOUS	8.2 KV	17.6 KV	SPONTANEOUS	8.2 KV	17.6 KV
0	1523908	385727	9220	1523907.9980	385727.0385	9220.4634
1	74	158	58	79.9960	188.9148	92.0760
2	5	25	19	.0021	.0462	.4598
3	1	6	16	.0000	.0000	.0015

* The average total X-ray dosage for the 17.6 kv is considerably higher than that for the 8.2 kv group. This fact is reflected in the proportionately greater number of variants observed.

It has been seen that multiple mutations occur at a frequency considerably higher than that expected by chance. The question may arise as to whether or not this increased frequency is alike in each of the three groups —spontaneous, 8.2 kv, and 17.6 kv irradiation. The comparison of the mutation frequencies of spontaneous and 8.2 kv mutation gives a probability of similarity between them of 0.05. The comparison of the spontaneous group with the 17.6 kv group gives a probability of considerably less than

one in 1,000 that they are the same in the distribution of their mutants. A similar comparison of the 8.2 kv and 17.6 kv X-ray groups shows these groups also to be distinctly dissimilar. This dissimilarity occurs principally in the increase of double and triple mutations within individual bacteria in the higher voltage group. Since a greater amount of energy is available to the photoelectron of high voltage as compared with those of low voltage, it would seem to follow that the absorption of such high energies in the bacteria favor the mutation of two or more genes within the organism. It is possible that in the wave lengths used in these studies there is a region comparable to that between the X-rays and the ultra-violet, as demonstrated in Drosophila, where the energy of X-rays is sufficient to induce large numbers of translocations and deficiencies as well as intra-molecular changes, while in contrast, ultra-violet irradiation is sufficient to induce only intra-molecular changes.

To explain the higher frequencies of double and triple mutations observed in both the spontaneous and X-ray groups it is necessary to invoke other hypotheses than those already mentioned. Three such hypotheses suggest themselves.

(1) A single mutation in hereditary material may change the expression of two or more phenotypic characters.

(2) In the mechanics of the mutation process a single event capable of producing a mutation may carry over to two or three genes instead of just one.

(3) A mutation when observed causes the observer unconsciously to examine that colony with extreme care, and more changes may be noted than otherwise would be the case.

The discussion of these hypotheses is complicated by the high frequency of the 0 class.

In view of the many known cases of multiple effects of genes in higher organisms, the first hypothesis would appear to be a reasonable explanation. In Drosophila and other organisms, many genes are known that affect the characteristics of two or more organs. It long has been recognized in bacteria that changes in virulence, antigenic properties, and other characteristics often accompany or are associated with the change from rough to smooth colony type. Such associated changes could be due to the multiple effects of a single gene. This explanation is not entirely satisfying, however, since in our experiments each of the characters studied ordinarily arises as a single change.

The second hypothesis has possibilities of two or more subsidiary hypotheses. In table 5 are listed the expected frequencies of no, one, two, and three mutations per bacterium on the assumption, expressed earlier in this paper, that the appearance of one mutation is independent of another in

each of the groups. It is obvious that the corresponding expected curves do not fit the actual data in any of the three groups—spontaneous, 8.2 kv, and 17.6 kv X-ray. In each case the proportion of double and triple mutations is too large. This observation would appeal to those who interpret mutational effects of X-rays as dependent upon the surrounding medium being first activated, the excitation causing lability of the genes enveloped by the activated medium. Should such an action take place, it would be fairly easy to account for the higher frequency of two and three character changes. On the other hand, were this true, the X-ray treatments would be expected to show proportionately higher numbers of multiple mutations than the spontaneous group, since X-rays of the order used have ample electronic energy to cause such chain reactions. Just the opposite result was observed, however. In the spontaneous data five double mutations were observed (not including triple mutations) where the expectation is only 0.0021, or the rate is 2,380 times expectation. With X-rays of 8.2 kv there are 25 doubles when 0.046 were expected or the rate is 534 times expected. For the 17.6 kv X-rays there are 19 double variants where 0.46 are expected, a rate 41 times expectation. The spontaneous rate is 58 times greater than the 17.6 kv X-ray rate, thus militating against this interpretation.

But this argument does not entirely settle the question, for the data may be interpreted from a different viewpoint, a viewpoint which is no less real than that presented above. Mutation in a bacterial cell could be due to two or more independent causes—that is, cells may be sensitized by one agent so that another agent is then able to produce a mutation in greater frequency than in an unsensitized cell. The course of such chain causes leading to mutations could be illustrated by X-ray effects. It might be assumed that in order to mutate at all the cell had to absorb a photoelectron and then become sensitive. But whether or not the cell would mutate again in any of its genes would depend on the direction of the ion path traced by the photoelectron. For the purposes of this problem we should look only to the cells which had mutated at least once and then compare the actual rates for two or more mutations with those expected on chance. Data for the spontaneous group show 80 cells which contained at least one mutation. The mean frequency for further mutations is 7/80 or 8.75 percent. The expected chance distribution for these cells then is 73.4 cells with one, 6.3 with two, and 0.3 with three mutations. This distribution agrees very well with the observed. It would seem that once the untreated cell is prepared for mutation, repeated mutation could be attributed to some agency acting by chance.

The X-ray data, when analyzed in like manner, go counter to this interpretation. The binomial equation attempts to compensate for the large

numbers of triple mutations by too low a number of single and too a great a number of double mutations. The expected numbers even then fail to reach the observed frequency of triple mutations. This evidence would favor the view that the agency making for the original mutations in itself tends to increase further mutations as might be true if two or more mutations came from a single ion tract. This view is further borne out by the higher frequency of triple mutations in the 17.6 kv irradiations where higher roentgen exposures were used and the tract of the photoelectrons is longer.

The data give only an inconclusive analysis of this second hypothesis. However, they do suggest one other point. The mechanism of mutation for the spontaneous group appears different from that of the X-ray group. Since radiant energy was used in the X-ray group, this suggests that radiant energy—that is, in the form of cosmic rays—is not responsible for the mutation in the spontaneous mutation group, a conclusion which would agree with the present viewpoint on mutations in higher animals.

The third hypothesis is difficult to evaluate. All colonies were carefully examined and any bias certainly is not intentional.

MUTATION IN A14 STOCK

The distribution of the different mutant types found in A14 stock is presented in table 2. This stock is characterized by large, spreading, smooth, mucoid, diffusely gray-colored colonies. In contrast to S15, A14 is pale yellow, smooth colony type. Table 2 shows that A14 may mutate from this pale yellow type to either the dark yellow or the white. It also may mutate from the smooth to the rough forms. In fact, the most frequent variants are either a rough mucoid type, in which the colony appears rough for about the first two days of growth and then forms a smooth halo around this rough center, or small, darker-yellow smooth mutants that form a dry compact mucoid colony. A single mutation from the mucoid type to the non-mucoid was observed.

It may be of interest to note that not all the variants are of equal viability. A small transparent colony type that was isolated from A14 proved to be difficult to keep alive on culture media. This forms another point of similarity between the variants in the bacteria and those of Drosophila, for many of the Drosophila variants are not so viable as the parent types.

The results with S15 and A14 stocks make it evident that *Ph. stewartii* can change from dark yellow to pale yellow or to white or from pale yellow to either dark yellow or white. It may also change from rough to smooth or from smooth to rough, from non-mucoid to a mucoid growth or in the opposite direction. The variant changes therefore may be direct or in the reverse direction, depending on the parent type employed in the given study.

The observed changes in this species of bacteria are thus comparable with those which are noted in Drosophila where the mutations may be direct from the wild type to a mutant or in the reverse direction.

The data on A14 as found in table 2 are not very extensive. There are, however, two points of significance. The ratio of mutant to normal cells found in A14 is twice the ratio found in S15. Of 156,000 colonies observed without treatment 18 mutations were found (0.0116 percent). For S15, where ten times this number of colonies were observed, 92 mutations were found (0.0061 percent).

Again in A14, as with S15, it was found that multiple mutation occurred much more frequently than expected. Of the 25 mutant colonies observed after X-ray treatment of the A14 stock, four exhibited mutation in two characters. In the spontaneous groups all 18 mutants had changed in one character only.

MUTATION AND VIRULENCE

Several of the X-ray-induced variants showing pronounced morphological changes were tested for a possible change in virulence associated with the morphological change on which the mutant was classified. These mutants were tested on susceptible maize seedlings (Inbred GB797), at the growth stage when two to three leaves had expanded on the seedling, by inoculating hypodermically through the first node with a heavy suspension of the test bacteria. Green weights of each group of plants were taken 14 days after inoculation and virulence index calculated.

$$100-\frac{\text{Green weight of test plants}\times 100}{\text{Green weight of check plants}}$$

The indices may vary from zero to 100, the index increasing as virulence of the bacteria increases. The virulence indices for the different mutants tested are given in Table 6. All strains produced definite lesions and typical symptoms of bacterial wilt.

These mutants were derived from the two parental stocks S15 and A14 as indicated. These stocks were widely different in virulence as well as in the characters noted in the previous table. The virulence index for S15 was 31 and that for A14 was 75. Most of the mutants of the weakly virulent S15 stock exceeded the parent culture in virulence, although they did not equal the virulent culture (A14) in this respect. In contrast, most of the mutants of the highly virulent A14 were less virulent than the parent but more so than the weakly virulent S15 culture. These results may be explained by the fact that each parent is an extreme type and only mutations away from the extreme can be detected easily. It may be that when the gene combination is such as to cause extremely low virulence, the

probability of a change toward increased virulence is greater than a change toward still lower virulence. With a highly virulent organism, such as A14, the converse is true.

There are certain apparent correlations of colony morphology and virulence (table 6). The change from a rough to a smooth form seems to favor an increase in the invasive power of the organism. In the S15 (400) series,

TABLE 6

Virulence indices of mutants

SOURCE AND CULTURE NUMBER	COLONY CHARACTERISTICS	VIRULENCE INDEX
From S15 stock		
Parent (400)	Dark yellow, Rough I	31
476	Pale yellow, Rough I	35
420	White, Rough I	28
427	Rough II	42
417	Rough III	30
457	Rough IV	45
429	Rough VI	49
441	Smooth	58
452	Smooth	62
467	Smooth	41
431	Smooth	70
479	Smooth, mucoid	56
458	Small	28
From A14 stock		
Parent (500)	Large smooth, undifferentiated center	75
502	Large smooth differentiated center	81
506		73
560	Small smooth compact dry	65
520	Small smooth compact dry	51
530	Small smooth compact dry	53
525	Rough mucoid	57
526	Rough	46
537	Small S, non-mucoid	53
509	White	78

smooth variants are more virulent than the rough parent or the rough variants. In the A14 (500) series, the large colony smooth variants retain the virulence of the parents, and the small compact dry type or the more rough colonies are less virulent. It is evident that mutations in morphology may be attended by either increase or decrease in virulence and that such changes are not uniformly in the direction of reduced or increased pathogenicity.

STABILITY OF MUTANTS

The mutation rate of observed variants has not been determined except in a preliminary way. An indication of the stability of the variants, however, is obtained from their behavior when kept as stock cultures. When they first were observed, the mutants were plated and their purity checked; they were then placed on nutrient dextrose agar slants and kept in a refrigerator at 7°C. These stock cultures were transferred every six weeks. When plated again after nine transfers, most cultures were pure or predominantly pure for the original variant type. A few cultures contained variants, but in most cases the predominant type was that of the original. It should be noted, however, that the number of colonies that were observed in these ninth transfer platings were not large, usually fewer than 750 per culture.

The unstable types or segregating variants are more difficult to hold as stock cultures because they also form one or more stable types which usually become predominant and replace the unstable variants. The eventual predominance of the stable type is to be expected, for this type is not only reproducing itself but is being added to by reproduction of the unstable type.

DISCUSSION

The variations in *Phytomonas stewartii* described in this paper were obtained by examining individual colonies, almost all of which had arisen from single cells. The cell originating these variants arose under the stimulus of X-rays of low quantum energy. Under the conditions of treatment used the intensity was such that 100,000,000 viable cells were reduced to about 1,000 in 25 minutes of irradiation. The types of variants that arose under the two treatments were similar, and these in turn were similar to the spontaneous variation that occurs during growth. Thus under both natural and X-ray conditions the following changes have been observed: in colony type from rough to smooth and from smooth to rough, from one rough colony type to another, from one smooth colony type to still another, from the mucoid colony type to the non-mucoid and the reverse, in alteration of the pigment intensity of the parent type to that of greater or lesser intensity for the variant, and in increased or decreased virulence. Such variations frequently are spoken of as dissociation and are considered by some to be one phase of a cyclogenic type of life cycle (HADLEY 1927). Since X-irradiation resulted in a much higher rate of mutation, we can be relatively certain that the variation stimulated by X-irradiation is mutation as commonly understood. Therefore, it appears logical that similar but spontaneous variation be likewise considered as

mutation. The terms "dissociant," "saltant," "variant," and "mutant" on this evidence might be considered synonymous and due to hereditary changes resulting from gene mutation.

Increasing the rate of mutation is the most striking effect of X-rays on variability. Except for mutations to an unstable form, the mutations are stable and heritable from parent to daughter cells and therefore must be considered direct changes in the germ plasm. Such variants have proven stable in a variety of environments and for many cell generations.

In addition to the effect of X-rays in initiating mutation, they also cause inactivation or death of the bacterial cell. Increasing the dosage of X-rays of all quantum energies increases the number of inactivated bacterial cells. Similarly, increasing the dosage also increases the rates of mutation for different phenotypic characters. It seems entirely probable that the differences between inactivation and mutation are only those related to the position in which the energy is absorbed by the genic substances. Since most genes are necessary for life, the destruction of the capacity of a gene to reproduce would in effect eliminate that gene, and if the organism were haploid, the individual. Similarly, if the energy were absorbed by a chemical bond not primarily important to reproduction, the gene structure might be altered, thus leading to a visible mutation without lethally affecting the reproductive capacity of the cell.

Considerable evidence secured from this study indicates a basic similarity in the mechanism and the physical basis of inheritance between groups as widely divergent as *Ph. stewartii* and *Drosophila melanogaster*. The mutation rates observed for *Ph. stewartii* are of about the same order of frequency on a roentgen basis as the mutation rate in Drosophila. Certain differences in the frequency of appearance of the different mutations in the spontaneous and X-ray groups of *Ph. stewartii* further suggests this similarity. After X-ray treatment, unstable mutants have a much greater frequency than they do in the spontaneous group. This fact corresponds to a similar observation in Drosophila where X-rays markedly increase the unstable mutants as compared with stable mutants. These variegated types frequently are associated with breakage of the gene-bearing material, the chromosomes often being reorganized in a complex form. The observed increased frequency of such types in the irradiated bacteria suggests the possibility that in this form as in higher organisms there is some organization of the genic substances into larger entities comparable to chromosomes.

The spontaneous mutation rate of *Ph. stewartii* indicates a stability of the species comparable with that of the higher forms. Variants, when they appear, arise suddenly and in their final form. Variation appears to be

abrupt and discontinuous with no series of gradual changes between the parental type and that of the variant. This is equally true of the X-ray changes.

The individual characters in these bacteria generally mutate singly as they do in Drosophila. However, mutations of two or three characters in the same individual have been observed, and at a greater frequency than expected by chance in both the spontaneous and X-ray material. It has been argued that such multiple changes in Drosophila genes when exposed to X-rays are brought about by the particular direction the photoelectron takes through the inheritance substance. If the path traverses two or more genes, a double mutation would be produced. For these bacteria this argument would seem to be invalid, since we find that the relative frequency of these double changes is greater in the spontaneous mutation group than in those treated with X-rays. No explanation of this relationship is available at the moment.

The observed variation is classified readily into the broad groups of colony color, colony surface, or colony size. Within these groups, however, there are other distinct types. For example, in the S15 study, five rough types and three intensities of yellow pigmentation have been observed. The series of rough mutants pictured in both Plate I (A, B, C, D, E) and in Plate II (L, M, N) gives the impression of a graded series as does the colony color series—dark yellow, pale yellow, and white. A possible series of smooth mutants is not clear cut, but the impression gained from work with mutants of this species is that there is a series of almost infinitely small differences from a uniformly non-chromogenic smooth type to a uniformly chromogenic type. The intermediate types of this series are initially chromogenic and with later growth of the colony form non-chromogenic material around this center (see "smooth" colonies Plates I and II). Smoothness and roughness may be a distinction indicating different stages of the same series. The similarity of these described series to the classical allelic gene series of Drosophila eye color, rodent coat color, and anthocyanin color in maize or primula is striking, but since similar series can be arranged for characters due to non-allelic genes, it cannot of itself be considered proof of allelism.

Most mutations of morphological characters observed in this study were associated with certain changes of function. This is evidenced by the fact that the virulence of the mutants caused by X-rays may be increased or decreased. It is of interest to note, however, that the virulence of the weakly pathogenic strain used in this study was more frequently increased than decreased by mutation, whereas the virulence of the highly pathogenic strain was more frequently decreased than increased. The observed series of discontinuous changes in virulence shows that definite genes con-

trol the pathogenicity of *Ph. stewartii* for maize. With this species McNew (1940) and Lincoln (1940) have described rather complete series of virulence change that range from avirulence to high virulence. Such series may be the expression of multiple alleles, of multiple factors, or interaction of some other type.

The evidence herein presented indicates strongly a strict gene basis for heredity in the bacteria as in other organisms. As is well known, this inheritance affects many physiological as well as color or morphological characters. Strains differ in their disease causing ability or enzyme forming capacity for the fermentation of sugars or breaking down of proteins, etc. The work herein presented favors the view that each of these changes is controlled by individual genes.

SUMMARY

This paper presents a comparative study of mutations in *Phytomonas stewartii* under natural conditions and under the stimulus of X-irradiation of low quantum energy. Irradiation was at an intensity such that 100,000,000 viable cells suspended in broth were reduced to about 1,000 in 25 minutes of treatment. Survival during irradiation follows the simple exponential function—survival ratio $= e^{-ar}$. Mutations were observed in colony color, surface appearance, and size. The rate of mutation following X-irradiation is greater than the observed spontaneous rate. X-ray-induced mutation differs from spontaneous mutation only in the frequency of occurrence, no differences in the kind of mutation produced being observed. The pattern of mutation observed in two widely different stocks of *Ph. stewartii* was similar. Mutations of colony characters may be accompanied by either increased or decreased virulence for maize. Except for mutations to an unstable form, mutants appear to be as stable as the parent strain from which they were derived. From evidence secured in this study it would appear that the terms "mutant," "variant," "saltant," and "dissociant" as applied to bacteria are synonymous and are applied to phenomena resulting from gene mutation. From this point of view the physical basis of inheritance of *Ph. stewartii* is similar to that of higher organisms.

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