VARIATION IN *STREPTOMYCES*

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The Organism

There can be no fundamental progress in our understanding of variation in *Streptomyces* until we know more about the organism itself. We need, first of all, to know whether or not the substratal and aerial mycelia are constitutionally different. This will require intensive studies in cytology and morphology, fields which can hardly be claimed to be enjoying a heyday at the present time in biology.

The proponents of a two-phase life cycle who postulate a haploid substratal mycelium and diploid aerial growth have been principally the European investigators Badian (1936), von Plotho (1940), and Klieneberger-Nobel (1947). Badian (1936) claimed that the chromatin is distributed through the hyphae as chromosomelike bodies which unite just before conidial formation, so that each conidium has a bivalent chromosome. When a conidium germinates, this bivalent chromosome undergoes two divisions, one of them a reductional division. At the same time, from one to three germ tubes arise, each of which receives one of the four daughter chromosomes. The remaining chromosome or chromosomes disintegrate.

Klieneberger-Nobel (1947), who has strongly supported the antithetic haploid-diploid life-cycle concept, believes that nuclear fusion occurs between filaments in the haploid, substratal, or primary mycelium. The fusion product is an initial cell which consists of a darkly staining nuclear body surrounded by cytoplasm and later enclosed by a cell wall. The initial cells produce the aerial or secondary mycelium.

Significantly, the point of view of Klieneberger-Nobel for a two-phase life cycle in *Streptomyces* has been applied to *Actinomyces bovis* by Morris (1951). Here he presents a haploid-diploid cycle in which there are *two* fusions and one reduction division. The fusions are described by him as follows:

"Nuclear material from the poles of cells in the 'A' phase moves to the center and fuses, during which process the cell contracts and begins to swell at one end, becoming oval or pear-shaped. If the adjacent cell remains unaltered, a drumstick appearance results. Two of these cells then conjugate, and a fusion cell is then formed in a manner reminiscent of that described by Klieneberger-Nobel (1947) for the initial cell of *Streptomyces*, and observed by the author in *Micromonospora*."

In line with the same general life-cycle point of view, Bisset (1950) has postulated nuclear fusions for *Mycobacterium* and *Corynebacterium*, and Webb *et al.* (1954) believe that in *Nocardia corallina* "nuclear process during fragmentation appears to give rise to binucleated bacillary cells. Coccoidal cells, however, are observed to be uninucleate."

Opposed to the two-phase life-cycle concept is the long accepted view that *Streptomyces* is an asexual organism in which the individual is differentiated

into a substratal portion specialized for vegetative development and an aerial portion differentiated for reproduction by conidia. An aerial filament, according to this view, may arise directly as a branch from any vegetative hypha at any point. The two are quite unlike in appearance, the aerial filament being considerably larger in diameter. Its walls are usually thicker and of a waxy consistency giving a "bloom" to the entire aerial growth.

However one regards the claims for a two-phase life-cycle in *Streptomyces*, the idea is before us and must be thoroughly tested by cytologists and morphologists. The evidence, at the present time, is altogether too meager to justify or refute the hypothesis. I strongly support Erikson (1947 and 1949) in his view that this problem is of major importance and "that in a discussion of variation, such as the frequent phenomenon of asporogenous sectors produced within a single colony, it is essential to consider the origin of the inoculum in every instance."

Let us examine some of the reported observations that relate to the two-phase life-cycle concept. First of all, are the aerial spores and substratal filaments genetically unlike? Supporting the idea is the careful work of Erikson (1948) on *Streptomyces coelicolor*, where he found that spores used as inocula frequently yielded permanent variants, whereas young vegetative filaments did not. Sister spores of the same chain gave dissimilar growths which remained so throughout 10 successive transplants. Opposed to the idea is the fact first established by Carvajal (1947) that typical spores of *Strepto-*

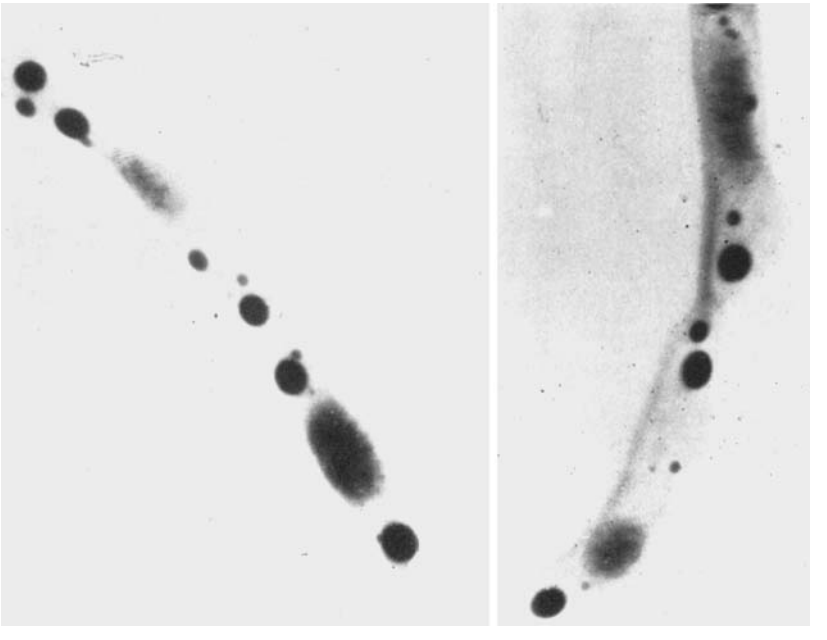

FIGURE 1. *Nocardia* (Proactinomyces) *ruber* grown on nitrogen-free agar at 28°C. Electron micrographs taken at 50 kv. by Professor N. M. McClung. The granules within the filaments are of unknown nature and functions.

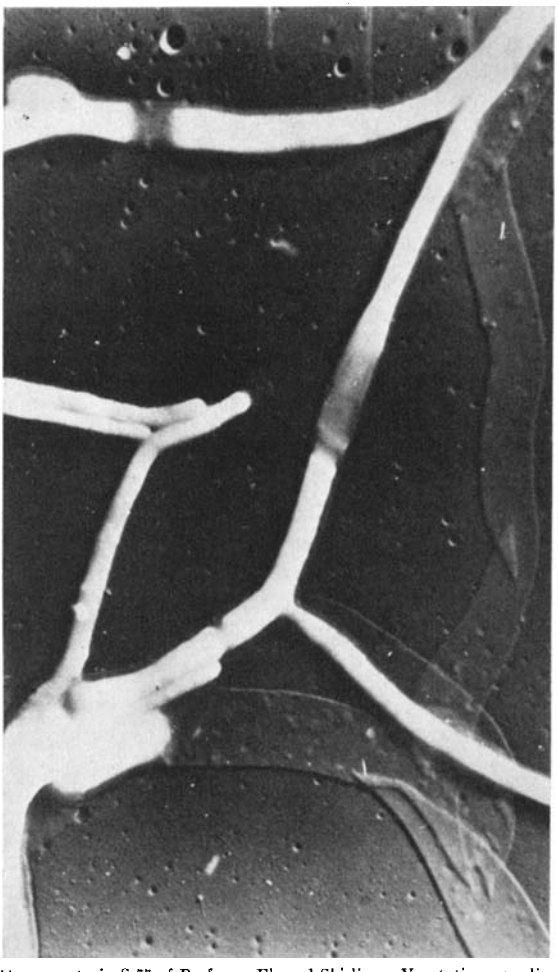

FIGURE 2. *Streptomyces* strain S-77 of Professor Elwood Shirling. Vegetative mycelium grown 28 hours in glycerol nutrient broth. Electron micrograph of chromium shadow-cast filaments of varying diameter, some partially or completely lysed.

myces are produced by substratal mycelia in submerged culture. He showed excellent photographs of characteristic spores formed even inside of the germ tubes of conidia which were of aerial origin. Pittenger and McCoy (1953), using ultraviolet rays as a mutagenic agent, found that the mutation frequency was essentially similar for spores formed in either aerial or substratal hyphae or for spores at incipient germination.

There are few observations testing possible fusions of filaments in *Streptomyces*. The author has observed that some isolates grown from single spores or filaments on agar blocks tend strongly to have their branches grow appressed to one another. In FIGURE 3 is shown a typical instance in which the ends of filaments tend to curve, permitting the terminal portions and branches to be

firmly aligned. In older mycelia, as many as six filaments in a column have oftentimes occurred. This strong tendency for filaments to grow as compact parallel strands would permit anastomosis and possible production of heterocaryons, or perhaps nuclear fusions. Carvajal (1946) published electron photomicrographs showing evidence of fusions between germ tubes and filaments of young cultures of *Streptomyces griseus*. Pontecorvo's (1953) remarkable success in producing heterocaryons in asexual fungi by anastomoses of filaments of contrasting genotypes suggests that similar studies be attempted on actinomycetes.

Erikson (1949), however, points out that the general absence of anastomoses is one of the characteristics of the group. He says he "has watched literally thousands of growing colonies under a great variety of cultural conditions, and it has been most instructive to note the way in which, when one filament comes in contact with another, it slides over, under or around the obstruction."

Before concluding my remarks on the organism, I should like to mention the fact that I have been somewhat impressed by the heterogenous appearance of the filaments of young substratal mycelium as viewed under the electron microscope. FIGURE 2, furnished through the courtesy of Doctor Elwood Shirling, is a characteristic example. The filaments vary greatly in diameter

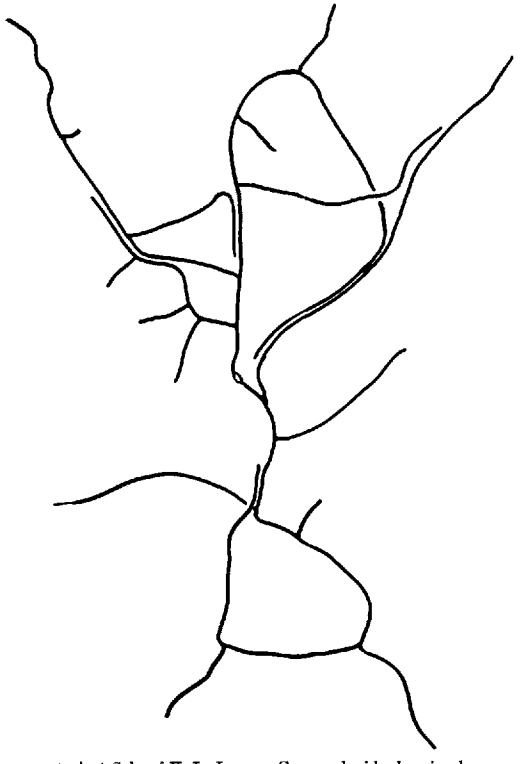

FIGURE 3. *Streptomyces* strain 1-Sch. of K. L. Jones. Camera lucida drawing by means of a 4-mm. objective and a 12.5 ocular of vegetative mycelium 49 hours after germination on glycerol agar block at 22°C.

and, though they were only a day old when photographed, they show small lysed portions as well as long ghost hyphae. I have not seen clear views of the interior of vegetative filaments of *Streptomyces*. McClung, however (FIGURE 1), found that, in *Nocardia*, it was possible to see internal structure in nitrogen-starved hyphae. Small dense spherical bodies of variable sizes alternated with a larger diffuse structure. Parallel studies of the same material under a light microscope confirmed the presence of the two types of granules. Neither could be identified as nuclear, since they were not seen in division, nor were Feulgen reactions consistent.

Others, *e.g.*, Carvajal (1946), Erikson (1949) and Webb *et al.* (1954), have commented on the heterogeneity of the internal organization of the aerial hyphae. Carvajal (1946) observed that spores may have one or more nuclei, and that "the number of nuclei is by no means always proportional to the size of the cell." Erikson (1949) states, "Granting that chromatinic bodies are embedded in the cytoplasm, the writer has frequently observed an irregular distribution of this material in the separate elements of sporing hyphae, as revealed by vital staining with methylene blue." Webb *et al.* (1954), who admitted that they make no attempt to follow the life cycle reported by Klieneberger-Nobel, found the "surface mycelia" of *S. griseus* to reveal cross-walls irregularly spaced along the hyphae, "with some units apparently containing several nuclei." Their one published illustration shows, very clearly, dark bodies of various sizes and shapes within the hyphae.

It is therefore possible that the internal heterogeneity of filaments and spores in *Streptomyces* leads to variability of one kind or another in subcultures grown from single spores or filaments. Growths from larger masses of inocula would tend to be more uniform.

Comments on Observations of Variability in Streptomyces

Temporary variations. Under temporary variations I shall mention, in turn, those resulting from (1) direct and immediate effect of the environment; (2) the amount or age of inoculum; (3) unknown causes apparently of an intrinsic nature which produce fluctuating variations; (4) the gradual effect of the medium leading to a change, usually a loss, of a characteristic.

Anyone beginning work on *Streptomycetes* is impressed by the profound influence of the medium and the cultural conditions on the characteristics of an isolate. Krainsky (1914) was a pioneer in championing the idea that the nature of the growth is here dependent on the substratum and that, for purposes of classification, synthetic media and standard conditions of culture are a *sine qua non*. Unfortunately, even renowned investigators do not always adhere to this principle, but employ such highly complex and ill-defined test substrates as potato plugs and yeast extract peptone agar.

I have expressed the view for some time (1940) that colony characteristics of isolates, grown on standard synthetic media under constant environmental conditions, should be given special consideration in the delineation of species. "I believe that colonies of *Streptomyces* possess convenient macroscopic characteristics of taxonomic validity that are not duplicated in larger and less definite growths on slopes and plates. These include size, shape, nature of margin,

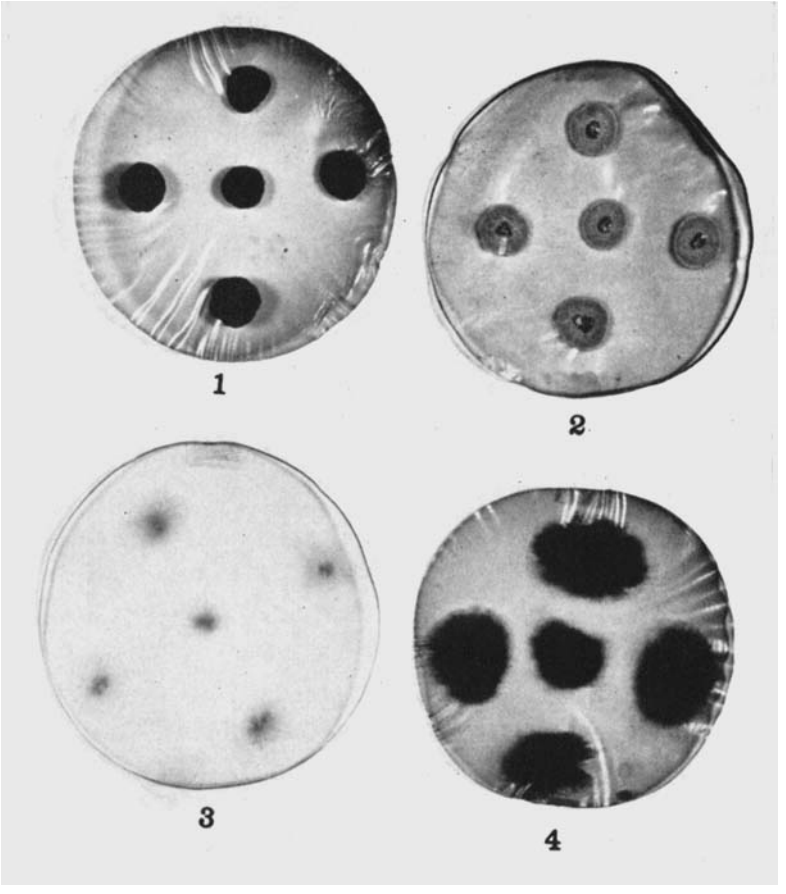

FIGURE 4. Herbarium specimens of colonies of *Streptomyces* to show diagnostic characteristics on synthetic media. The agar media were removed from the petri dishes after three weeks' growth of the colonies. The preparations were soaked in $HgCl_2$ solution to kill the colonies and to render the agar unfit for growth of molds, and they were then dried and mounted as permanent specimens.

topography position of the aerial mycelium, color of the colony and of the surrounding medium, and tendency to produce sectors" (Jones, 1950). As a practical consideration, I favor the preparation of permanent herbarium specimens of the agar films preserving the actual colonies on several synthetic media (Jones, 1950). See FIGURE 4.

It is not generally realized that the characteristics of a *Streptomyces*' isolate may be considerably altered by the medium on which it previously grew (FIGURE 5). The influence of the penultimate medium was first pointed out by Jones (1946) and Erikson (1947). This change is a temporary effect which needs further investigation on a biochemical level.

The quantity of inocula may affect pigment production and utilization of calcium malate even where vegetative mycelia alone is the inoculum (Jones,

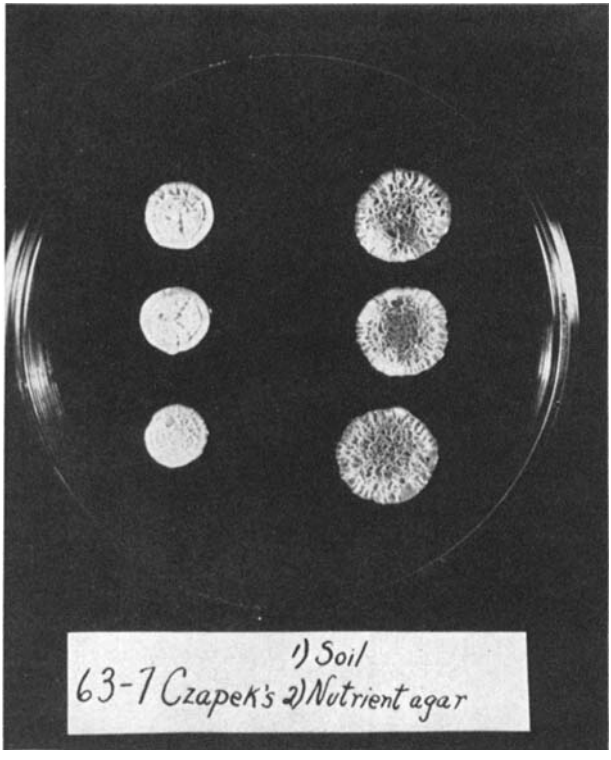

FIGURE 5. *Streptomyces* strain 63-7 of K. L. Jones, on Czapek's agar, three weeks' growth at 28°C. The effect of the penultimate medium is shown. Left row grew previously on sterilized soil and right row on nutrient agar.

1946). Erikson (1948) reported that "mass transfers of typical growth usually yielded uniform growth on plates." He found that, from a densely sporulating colony, 343 million spores were viable in each of five platinum loopfuls tested. In regard to the age of the inoculum, he stated in the same paper that stable permanent variants of *Streptomyces coelicolor* may arise from degenerate, aged vegetative mycelium, whereas parallel sets of nutrient glucose broth cultures, kept in vigorous condition by frequent subcultivation, remained essentially constant.

In certain isolates, particularly those producing diffusible pigments, a wide range of fluctuating variations may occur on each culture plate (FIGURE 6). When freshly isolated from the soil, an organism yields the entire range of variants in subcultures irrespective of the colony selected as inoculum. Fluctuating or continuous variations are apt to mislead observers to believe that the Streptomycetes are hopelessly labile. I encountered an extreme case in isolate 47–13 (1940 and 1946) which was established from a single spore but soon grew only as substratal mycelia. On Czapek's medium, it produced a greenish-black soluble pigment in some colonies. When the colonies first appeared, they were all colorless. After about six days, a pigment formed in

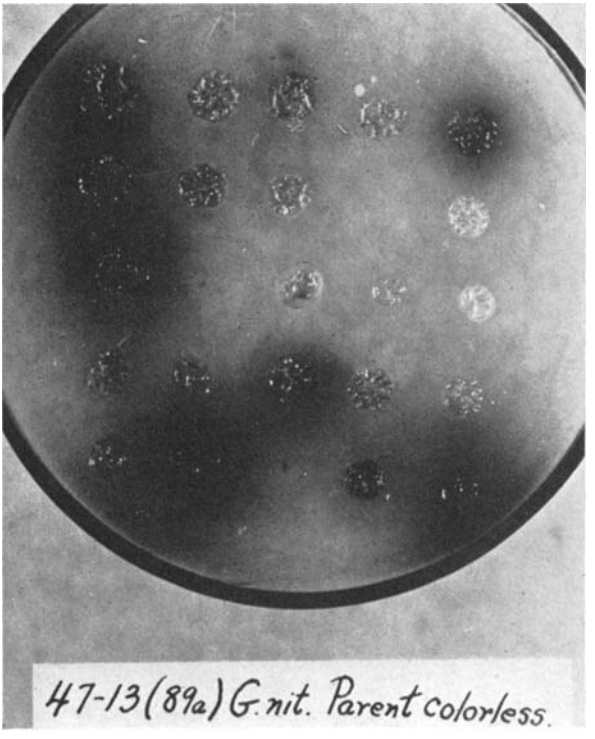

FIGURE 6. *Streptomyces* strain 47-13 of K. L. Jones grown on glycerol nitrate agar 28°C. three weeks. Fluctuating variation in pigment production is shown. The inoculum producing the colonies was colorless and asporous.

some colonies which gradually darkened and diffused. The concentration of the pigment varied, and some of the colonies remained colorless. Not only were there different intensities of the greenish-black color, but some colonies had a brown color or a mosaic of brown and greenish-black. Stanier (1942) encountered a widely fluctuating strain of *Streptomyces coelicolor* which he was culturing from spores, and he felt that the variations were probably attributable to intrinsic differences in the spores. He favored the cytological interpretations of Badian (1936) as an explanation.

Selection of colorless colonies in my asporous strain 47–13 led to a colorless line on glycerol nitrate agar after 31 months of culturing (see FIGURE 7). The result cannot be attributed solely to selection, however, as there was a gradual loss of pigmentation after prolonged cultivation on laboratory medium. Cultures transferred to soil regained their ability to produce pigmentation.

Lieske (1921) made particular mention of the gradual loss of characteristics in Actinomycetes, which seemed to him to be a widespread phenomenon in the group. In his day, it was the practice to speak of "dauermodifikation" or lasting modifications. Jollos (1921), who worked on the gradual adaptation of paramecium to the presence of arsenious acid, believed that changes occurred in the cytoplasm or in special autonomous structures of the cell. It is more

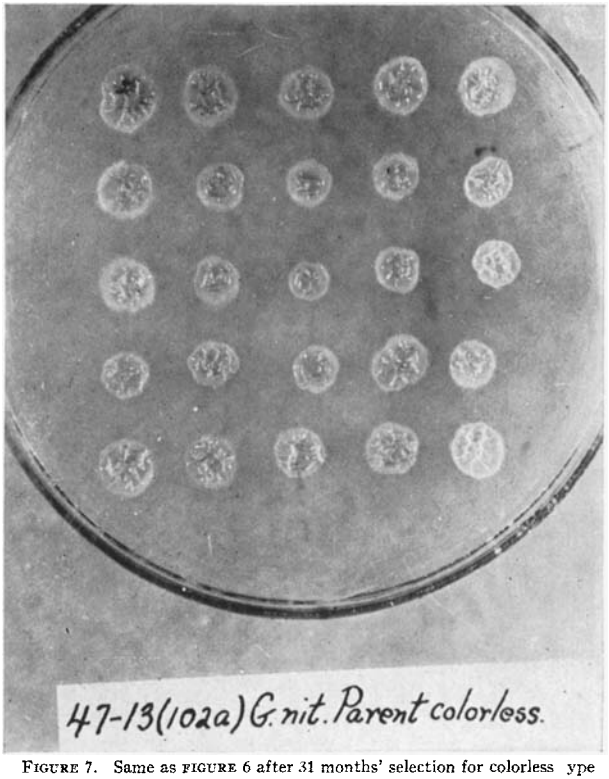

FIGURE 7. Same as FIGURE 6 after 31 months' selection for colorless ype

acceptable nowadays to approach the problem from the angle of enzyme induction and selection and mutation. We lack precise studies on the exploration of these possibilities.

Permanent variations. In considering permanent variations in *Streptomyces*, I should like to pay homage to Lieske (1921), who maintained over 100 isolates for many years, subculturing from single spores or filaments. In these isolates, he met with several permanent variations. A classical case was a 6-sectored colony in which each sector gave a different variant, the changes being in temperature requirements, odor, presence of spores, location of spores, color of spores, and diffusible pigments produced by the substratal mycelium.

Lieske was confident that the sectors he studied were not the result of any mixing of two or more kinds of inocula. He considered it possible that nuclear fusions occurred within the filaments, but doubted the actinomycetes were sexual in the sense the term was understood for higher organisms. The "colony" derived from a single spore was, to him, an individual. Sector formation suggested the vegetative sports or somatic mutations of higher plants. Erikson (1948) adeptly demonstrated that sectored colonies do not arise from two types of conidia. He mixed spores of (1) typical colored, agar liquefying

Streptomyces coelicolor with (2) colorless, nonagar liquefying variant, and grew colonies like (1) only.

Schaal (1944) clearly demonstrated that sectoring is especially associated with certain strains in the actinomycetes that produce potato scab. My observations on saprophytic soil forms confirm this. To bring out possible sector development, I suggest that the colonies be grown from single spores or at least from small amounts of inocula. The colonies should be widely spaced on the medium and allowed to develop under favorable conditions of temperature and humidity for at least a month. Skinner (Skinner, Emmons, and Tsuchiya, 1947) believes that "although variant sectors are sometimes seen, they do not seem to occur more frequently than they do in colonies of molds and bacteria of equal age." Schatz and Waksman (1945) showed that asporous variants of *Streptomyces griseus*, under certain cultural conditions, reverted to the parent sporulating strains.

It is well known that the rate of mutation can be increased by certain agents such as X radiation, ultraviolet radiation, and by nitrogen mustard ($Cl\ CH_2\ CH_2)_3N$. See, for example, Savage (1949), Dulaney, Ruger, and Hlavac (1949), and Pittenger and McCoy (1953). This finding has naturally been followed with particular interest by workers intending to exploit mutants that are profitable antibiotic or vitamin producers. Medium-specific mutants have occurred, as might be expected. Apparently no correlations have been found between morphologic type and antibiotic or vitamin yield.

Pittenger and McCoy (1953) have reported on an especially interesting investigation on induced mutations in *S. griseus*. "A spore suspension was in turn exposed to five successive treatments with heavy doses of ultraviolet, each exposure followed immediately by maximum photoreactivation with visible light," as recommended for cell restoration by Kelner (1949). In this way, a relatively high yield of variants in the surviving population was observed.

The role of phage in variation of *Streptomyces* has been studied by Carvajal (1953) and by Shirling (1953). Carvajal used phage on sensitive strains of *S. griseus* to produce a variety of colony types which were strikingly different in morphological, cultural, and biochemical characteristics. He considered the varying types among the surviving population to be mutants comparable to those induced by radiation and nitrogen mustard.

Shirling found that as many as 40 per cent of the fresh isolates of *Streptomyces* from a given soil were carrying phage. Lysis was evident only if widely spaced colonies were grown. Then a small percentage were soft, yeasty, asporous colonies. One remained true to type on subculturing and yielded a high titer of phage. If this phage were added to the normal type inocula, as many as 50 per cent of the colonies formed were soft.

Normal growth occurred when phage release was low. The electron microscope revealed some well-separated ghost sections in the mycelium. Soft colonies occurred when phage production was high. Ghost sections were then more numerous and more extensive, and surviving fragments were relatively short and easily separated. Segmentation also occurred with greater fre-

quency in soft types, and cell walls of ghost sections were probably more fragile. Free phage could be recovered from growths of the normal colonies but it was ineffective in increasing lysis if added to normal type inocula. Only soft colonies produced phage that was effective. Shirling considered the phage in the soft colonies to be a mutant of the less potent phage found in normal growths of his *Streptomyces* isolate.

Summary

The organism must be better understood before we can make any headway in comprehending variability in *Streptomyces*. First and foremost, an intensive cytological study is needed to give whatever direct information modern techniques can reveal on the finer structure of filaments and spores. Researches on developmental morphology will be of almost equal importance as the whole question of a two-phase life cycle must be settled one way or another. If definite nuclei exist, it may be possible to explore their genetical constitution by the production of heterocaryons through hyphal anastomoses.

In a decade hardly to be claimed as one of great distinction in morphology, it is disquieting to find life-history papers on the perplexing actinomycetes illustrated only by composite diagrams. We must publish photomicrographs and authentic drawings of particular observations. These are the basis for our ideas on nuclear behavior and sequential changes in morphology, and we must know how far each of us has progressed in acquiring raw data.

The fact of variability is so patent in *Streptomyces* that it behooves all investigators to bear it in mind. For meaningful taxonomic or genetical studies, standardized conditions must be rigidly maintained.

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