

# CYTOGENETIC STUDY OF *NOCARDIA CORALLINA*<sup>1, 2</sup>

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Cytogenetics has proved to be a valuable aid in the study of higher organisms but has received only limited attention in microbiology. Witkin (1951), Ryan *et al.* (1954) and Ryan and Wainwright (1954) have successfully applied cytogenetic approaches to the study of bacteria. Haploid and diploid cultures of *Escherichia coli* strain K-12 were studied cytologically by Lederberg *et al.* (1951) in an effort to establish genetic ploidy on a cytological basis. The comparisons revealed consistent and unequivocal differences in the nuclear appearance of these two forms but failed to explain the ploidy of the cultures on a cytological basis. Clark and Webb (1955a) studied the large cells of *Micrococcus aureus* cytologically and radiobiologically, and presented evidence for the diploid and triploid nature of these forms.

A cytogenetic study has been successfully applied to yeast by Lindgren (1949). The ploidy, life cycle, and sexual process have been substantiated by this approach although there is still some controversy in the field of yeast cytology. The success with which the limited application of cytogenetics has met in microbiology indicates that certain of the basic problems of microbial genetics may be profitably approached by using the available tools of this field. Webb (1956) has completely reviewed the literature on cytogenetic approaches involving microorganisms.

Preliminary cytological studies on *Nocardia*

*corallina* indicated that this organism possessed some characteristics well adapted to the testing of microbial cytogenetic procedures (Webb *et al.*, 1954; Webb, 1956). The existence of a unicellular, uninuclear coccoidal stage in the growth cycle indicated that the organism was adaptable to accurate radiation studies. Preliminary studies suggested that a life cycle might be associated with the alternation of coccoidal and hyphal stages, and the possibility of the existence of a sexual process was also suggested. The purpose of this investigation was the application of available techniques to the study of microbial cytogenetics, using *N. corallina* as the experimental organism.

## MATERIALS AND METHODS

Stock cultures of *N. corallina* strain 4273 of the American Type Culture Collection were maintained on nutrient agar containing one per cent fructose and incubated at 28–29 C. This medium yields cultures of coccoidal cells after 2 days of growth with little hyphal development. Nutrient agar was used in all cases in which hyphae were studied. Slide cultures for phase studies were prepared in nutrient agar on Shoemaker slides. The application of the slide culture technique is limited in growth cycle studies because of the strictly aerobic nature of *N. corallina*. Reduced oxygen tension tends to retard and inhibit fragmentation of the hyphae and has prevented a study of fragmentation by phase microscopy.

The crystal violet nuclear stain (Chance, 1952) as modified by Webb *et al.* (1954), and the thionine-SO<sub>2</sub> nuclear stain (DeLamater, 1951a) were used in the nuclear studies. In the thionine-SO<sub>2</sub> technique, cells were either fixed in Bouin's solution, Carnoy's fixative, osmium tetroxide vapors, or were left unfixed. Each fixative produced essentially the same cytological appearance as the unfixed cells. The preparations were examined and photographed as temporary water mounts, or were mounted in the medium of Minsavage (1955).

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Azure A was substituted for thionine in some of the studies (DeLamater, 1951a). Azure A-SO<sub>2</sub> is highly specific for the deoxyribonucleic acid components of the cell and leaves the cytoplasm and cell wall completely unstained. In order to determine cell boundaries, the smears were counterstained for one minute in 0.05 per cent aqueous safranin.

A modification of the tannic acid-violet cell wall stain (Webb, 1954) was used in the cell wall and cross septation studies.

American Optical dark contrast medium and B minus contrast medium objectives were used in the phase microscope studies.

In X-ray studies, a dose of 1,500 r per min as measured with a Victoreen 250 r dosimeter was used. In these experiments, the cells were suspended and irradiated in saline containing approximately  $3 \times 10^8$  cells per ml. Duplicate 0.1 ml portions were removed at intervals, appropriately diluted, and plated in triplicate on nutrient agar. The plates were incubated 4 days at 28–29 C before the colonies were counted.

In order to eliminate misleading results caused by clumping, which is often extreme in *N. corallina*, cell suspensions were vigorously shaken with glass beads on a vibratory shaker, then subjected to differential centrifugation to remove the remaining clumps. The resultant suspension was examined with dark phase contrast to determine the extent of clumping. No suspension with less than 90 per cent single celled units was used in a radiation experiment except for the hyphal studies. The suspensions were also checked for cross septation, multinucleate and involution forms, and were eliminated if such existed.

## RESULTS AND DISCUSSION

*Growth cycle studies.* (1) Cytology:—Nuclear structure and behavior during the growth cycle of *N. corallina* as revealed by the thionine-SO<sub>2</sub> nuclear stain were similar to that revealed by the crystal violet nuclear stain (Webb *et al.*, 1954). These structures showed behavioral characteristics comparable to the nuclei of higher organisms. The typical nucleus of resting coccoids was vesicular and often had 5 or 6 spots arranged around the periphery of the vesicle as revealed by the thionine-SO<sub>2</sub> stain and phase microscopy (figures 1 and 2). These figures were less completely resolved by the crystal violet technique, but the general appearance was similar (figure 3).

The coccoids were observed to germinate by a swelling and reorganization of the nuclear material followed by an outgrowth from one or both ends of the cell (figures 4–7). The nuclear behavior during germination was suggestive of a reduction division, but classical meiotic figures were not observed, and other evidence indicates that this division is not reductional. The nucleus divided twice, which resulted in 4 nuclei in the elongated cell (figure 8). Some or all of the nuclei divided again resulting in hyphae with 6–8 evenly spaced nuclei (figures 8–10). These nuclear divisions did not initiate cross wall formation, and the hyphae remained coenocytic until just prior to fragmentation (figure 11). Stained preparations of hyphae with 8–11 hr of growth usually failed to reveal discrete spherical nuclei with either technique (figures 12 and 13). This nondiscrete stage probably occurred through the metabolic breakdown of the nuclear membrane. However, the possibility remains that the appear-

*Figure 1.* Thionine-SO<sub>2</sub> stain of 5-day-old coccoidal cells. 3,200 ×.

*Figure 2.* Phase study of 5-day-old coccoidal cells. 4,000 ×.

*Figure 3.* Crystal violet stain of 5-day-old coccoidal cells. 3,200 ×.

*Figure 4.* Thionine-SO<sub>2</sub> stain of 3½ hr germinating coccoidal cells. 4,000 ×.

*Figure 5.* Thionine-SO<sub>2</sub> stain of 2½ hr germinating coccoidal cells. 4,000 ×.

*Figure 6.* Crystal violet stain of 4 hr germinating coccoidal cells. 3,200 ×.

*Figure 7.* Crystal violet stain of 5 hr germinating coccoidal cells. 3,200 ×.

*Figure 8.* Thionine-SO<sub>2</sub> stain of 6 hr hypha. 3,200 ×.

*Figure 9.* Crystal violet stain of 8 hr hyphae. 3,200 ×.

*Figure 10.* Thionine-SO<sub>2</sub> stain of 7 hr hyphae. 3,000 ×.

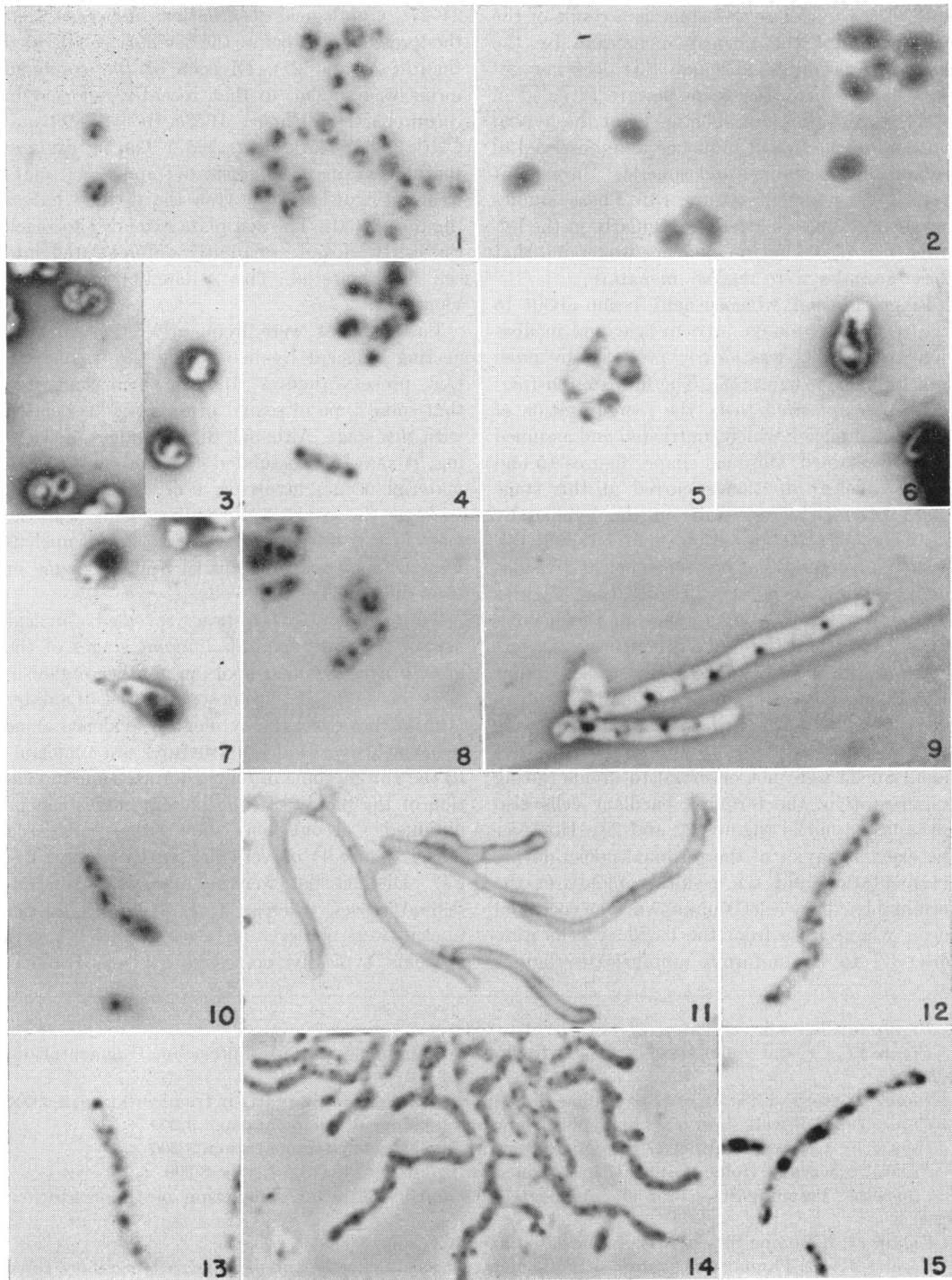
*Figure 11.* Cell wall stain of 12 hr hyphae. 3,200 ×.

*Figure 12.* Thionine-SO<sub>2</sub> stain of 11 hr hypha showing nondiscrete nuclei. 3,200 ×.

*Figure 13.* Thionine-SO<sub>2</sub> stain of 11 hr hypha showing discrete nucleus at hyphal tip. 3,200 ×.

*Figure 14.* Phase study of 15 hr hyphae. 3,000 ×.

*Figure 15.* Thionine-SO<sub>2</sub> stain of 12 hr hypha showing condensed nuclei. 3,200 ×.



*Figures 1-15*

ance of the nuclei at this stage is a result of the destruction of the nuclear membrane by the staining procedures, although this does not appear likely since discrete nuclei are revealed in other stages of growth. The nuclei at the hyphal tips and in the tips of branches were observed to remain deeply stained and spherical throughout hyphal development (figure 13). Phase studies revealed granules scattered irregularly in the hyphae (figure 14), but no evidence was found that these granules were nuclear in nature.

Fragmentation, which usually began about 15 hr after inoculation on nutrient agar and incubation at 28–30 C was clearly preceded by cross wall formation (figure 18). The first step in fragmentation appeared to be the reorganization of the hyphal nuclei, which contracted and assumed a deeply stained, elliptical shape (figures 15 and 16). A nuclear division occurred at this stage which produced cross walls in the hyphae by means of cell plate formation (figures 17 and 18). It should be noted that previous nuclear divisions did not result in cross wall formation. Figures 18–20 show fragmenting hyphae in which cross walls may be clearly observed.

The fragmentation process gave rise to binucleated bacillary cells (figures 22 and 23) which often remained attached in the form of segmented hyphae (figures 18 and 21). As the terminal nuclei were not observed to divide during fragmentation, the terminal bacillary cells also contained 2 nuclei (figures 22 and 23). However the exact behavior of the terminal nuclei during fragmentation and the resulting ploidy of the terminal bacillary cells is unknown. The coccoidal forms which arose from the bacillary cells were observed to be uniformly uninucleate (figures

24–27). Cytological observations indicated that the 2 nuclei fused before the bacillary cell divided (figures 26 and 27). Division of the coccoidal forms was similar to that recently reported in certain bacteria (Chance, 1953*a*, 1953*b*; Webb and Clark, 1954; Clark *et al.*, 1957). During division the nuclear material separated and a cell plate was distinguishable between the divided halves (figures 28–30). The cell plate extended to reach the cell wall and apparently differentiated into cell wall material. The 2 daughter cells later separated.

Fusion tubes were frequently observed connecting adjacent hyphae during the fragmentation process (figures 31–33) which suggested that some type of sexual process was associated with this stage. Although direct evidence is lacking, it may be postulated that an exchange of material occurs involving 1 of the 2 nuclei in each of the connected bacillary cells. On the basis of this interpretation the coccoidal nucleus may arise by fusion of nuclei from the same or from different bacillary cells.

The thionine-SO<sub>2</sub> stain revealed nuclear "spots" or short bars at different stages of the growth cycle. Similar spots in another organism have been called chromosomes by DeLamater (1951*b*); however, the cytological evidence alone is inconclusive. If these structures are analogous to the chromosome of higher forms, some indication of the ploidy of the different stages can be obtained. Coccoids may show either condensed nuclei (figure 1) or vesicular nuclei (figures 24–27). Distinct spots were not observed in the condensed nuclei, whereas 3, 4, 5 or 6 spots per nucleus were observed in vesicular nuclei. Young coccoids typically possessed condensed nuclei

*Figure 16.* Thionine-SO<sub>2</sub> stain of 13 hr hypha. 3,200 ×.

*Figure 17.* Crystal violet stain of 13½ hr hyphae showing nuclear division preceding fragmentation. 3,200 ×.

*Figure 18.* Cell wall stain of 14 hr hyphae showing cross wall formation early in fragmentation, 3,200 ×

*Figure 19.* Cell wall stain of 16 hr hyphae showing late stage of fragmentation. 3,200 ×.

*Figure 20.* Cell wall stain of 17 hr hyphae showing late stage of fragmentation. 3,200 ×.

*Figure 21.* Crystal violet stain of 17 hr hypha showing chain of bacillary cells. 3,200 ×.

*Figure 22.* Thionine-SO<sub>2</sub> stain of binucleated bacillary cells after completion of fragmentation. 3,200 ×.

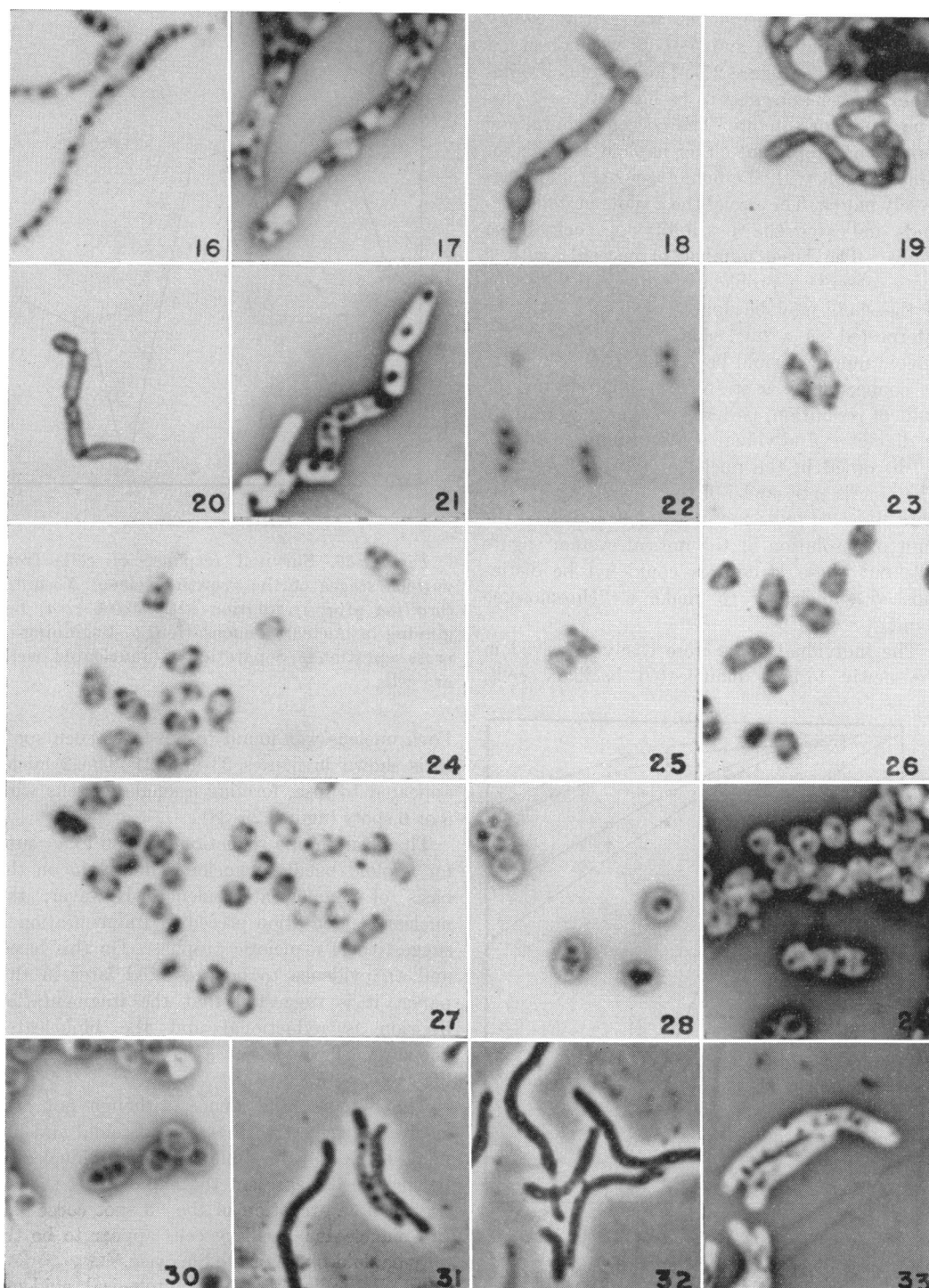
*Figure 23.* Thionine-SO<sub>2</sub> stain of binucleated bacillary cells. 4,000 ×.

*Figures 24–27.* Thionine SO<sub>2</sub> stain of uninucleated coccoidal cells and binucleated bacillary cells. 4,000 ×.

*Figures 28–30.* Crystal violet stain of dividing coccoidal cells showing cell plate. 3,200 ×.

*Figures 31–32.* Phase studies of 14 hr hyphae showing hyphal fusion. 2,500 ×.

*Figure 33.* Crystal violet stain of 14 hr hyphae showing hyphal fusion. 3,200 ×.



Figures 16-33

which became vesicular in the resting stage. Nuclei containing 3 and 5 spots were observed most frequently (figure 27). The haploid chromosome number appeared to be either 3, or 2 plus a nucleolus or satellite. The coccoids containing 3 spots may represent either haploid coccoids, or diploid forms with the homologous chromosomes closely paired. The size of the 3 spots in some coccoids indicates the possibility of their being double. The large number of coccoids with 5 spots presents a problem of interpretation. One of the spots may be double or 1 spot could be interpreted as a nucleolus, in which case the diploid number would be 4. The spots seemed to be connected by a strand somewhat below the limit of resolution, perhaps on the order of  $0.1 \mu$  in diameter. Individual spots usually could not be discerned in the nuclei of the hyphal stage. The nondiscrete nuclei of the developing hyphae were observed to be composed of strands near the limit of resolution of the optical system (figure 12), but these structures could not be distinguished sufficiently to make a "chromosome count."

The individual spots were easily observed in the newly formed binucleated bacillary cells.

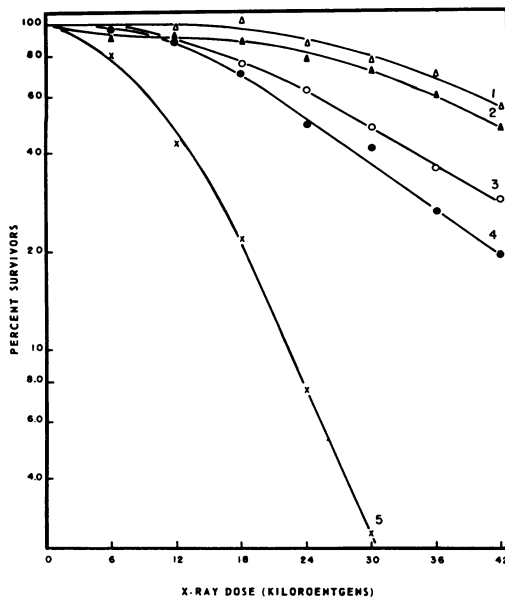


Figure 34. X-ray dose-survival responses of cells from various growth cycle stages of *Nocardia corallina*. 1,  $7\frac{1}{4}$  hr hyphae; 2, 12 hr hyphae; 3,  $3\frac{1}{4}$  hr germinating coccoids; 4, 7 day coccoidal cells; 5,  $25\frac{1}{2}$  hr bacillary cells.

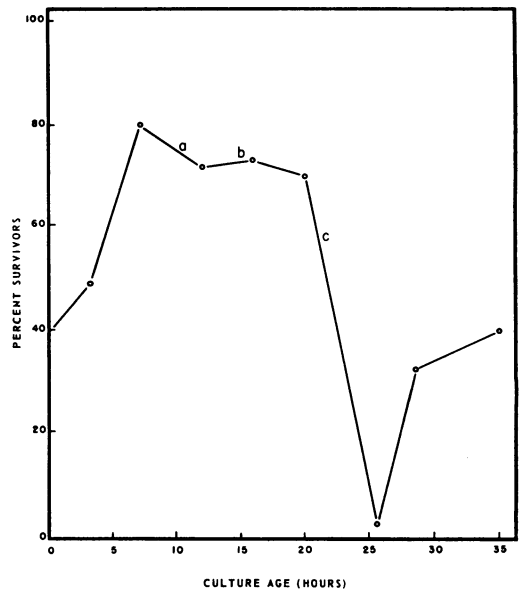


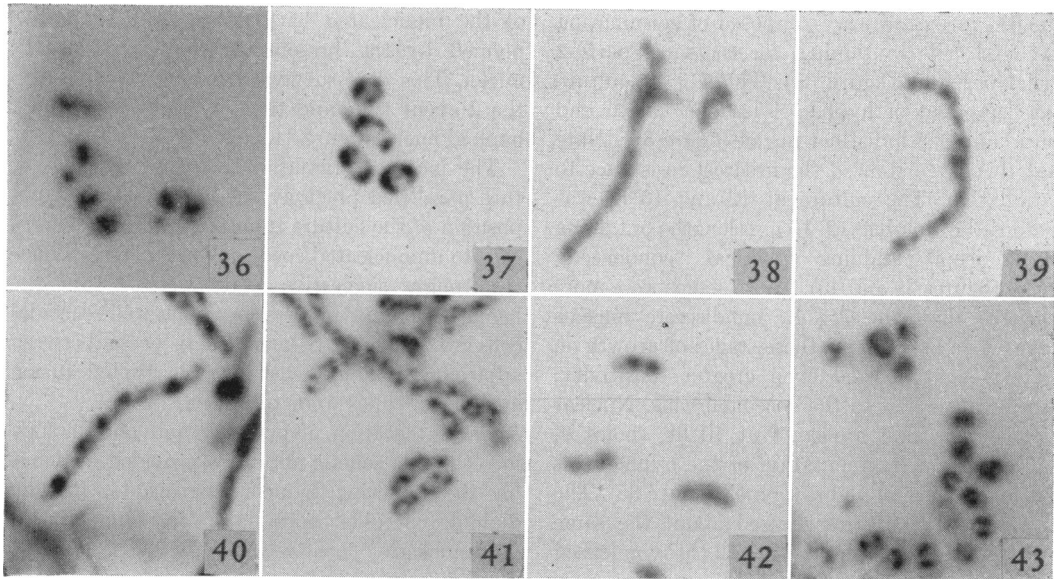
Figure 35. Survival response of cells from various stages of the growth cycle of *Nocardia corallina* after irradiation with 30,000 r. a, Beginning of nuclear condensation; b, Beginning of cross septation; c, Separation of binucleated bacillary cells.

Each nucleus was found to possess 3 such spots as is shown in figures 23 and 24. The 2 nuclei appeared to fuse, forming a single nucleus with 5 or 6 spots (figures 25-28).

The position of a reduction division to account for haploid bacillary nuclei is uncertain on the basis of cytological evidence. However, the nuclear condensation preceding fragmentation is suggestive of a meiotic prophase. On this basis, and on evidence to be presented later in this paper, it is suggested that the fragmentation division is reductional and the binucleated bacillary cells represent a haploid phase of the life cycle.

The cytological evidence, although not conclusive, is suggestive that the coccoidal and hyphal stages may be diploid and the binucleated bacillary cells represent the haploid stage. With the possible exception of the "3 spot coccoids," the binucleated bacillary cells appear to be the only haploid phase of the life cycle.

(2) Radiobiology:—The cytological evidence for a life cycle in *N. corallina* is at best only suggestive. Radiation studies were made in conjunction with cytological observations for the



Figures 36-43

Figure 36. Five-day-old coccoids. 3,200 X.

Figure 37. Three and one-fourth hr germinating coccoids. 3,200 X.

Figure 38. Seven and one-fourth hr hypha. 3,200 X.

Figure 39. Twelve hr hypha. 3,200 X.

Figure 40. Sixteen hr hypha. 3,200 X.

Figure 41. Twenty hr hyphae. 3,200 X.

Figure 42. Twenty-five and one-half hr bacillary cells. 3,200 X.

Figure 43. Thirty-six hr coccoids. 3,200 X.

purpose of further elucidating the basic cytogenetic problem.

Many factors may influence the response of cells to inactivation by radiations and contradictory radiation results, current in the literature, may be due in part to inadequate control or recognition of these factors. It has been shown, however, that the ploidy of a microorganism can affect the response to radiation (Latarjet and Ephrussi, 1949; Zirkle, 1952; Tobias, 1952). It must be recognized that the form of the radiation dose-survivor curve as such may mean very little. The shape may correspond to the number of cells per morphological unit, the number of nuclei per cell, the ploidy of the cell, or other factors. Thus morphological units and clumps must be determined before interpretation of radiation inactivation results (Clark and Webb, 1955b; Atwood and Norman, 1949).

X-ray experiments with *N. corallina* demonstrated that the unicellular, uninuclear coccoidal cells yielded a sigmoid or multihit dose-survivor response and could sustain radiation induced,

unpaired defects (Clark and Webb, 1957). These findings are in harmony with the cytological observations suggesting that the coccoids are diploid.

Different stages of the growth cycle of *N. corallina* were studied by means of X-ray dose-survivor experiments. In this series, a single, well aerated broth culture was used; however, tests showed that growth from a solid medium produced similar results. Aeration and agitation were obtained by placing a 500 ml Erlenmeyer flask, containing 100 ml of medium, on a Burrel wrist-action shaker. The standard X-ray irradiation procedure was used. The culture was sampled and the X-ray dose-survivor response checked at various cultural ages. The results of the series are given in figure 34. The resistance increased through 7¼ hr after which the culture became more sensitive. The per cent survival of stages during the life cycle at a constant X-ray dose of 30,000 r is given in figure 35. The cytological appearance of the culture at each growth cycle stage of figure 35 is given in figures 36-43.

The  $3\frac{1}{4}$  hr culture was composed of germinating coccoidal cells containing, for the most part, 2 nuclei per unit (figure 37). The  $7\frac{1}{4}$  hr culture was composed of hyphae 8–14  $\mu$  in length and containing 6–8 indistinct nuclei (figure 38). Note that this stage showed the greatest resistance to X-radiation. The culture at 12 and 16 hr was composed of hyphae 12–16  $\mu$  in length containing 10–12 deeply staining spherical "condensed" nuclei (figures 39 and 40). These stages were more sensitive than the  $7\frac{1}{4}$  hr nondiscrete nucleus stage. X-ray studies of these stages of growth on nutrient agar showed even greater sensitivity, very nearly equal to the coccoidal cells. Nuclear condensation first appeared at 10 hr, (point *a*, figure 35) and fragmentation of the hyphae was first in evidence at 15 hr, (point *b*, figure 35). The 16 and 21 hr cultures showed about the same X-radiation response as the 12 hr condensed nucleus hyphae. Figure 41 reveals extensive fragmentation at this stage. The culture was composed almost entirely of binucleated bacillary cells at  $25\frac{1}{2}$  hr (figure 42). A comparison of the X-radiation response of the bacillary stage with the  $3\frac{1}{4}$  hr binucleated germinating coccoids reveals a striking difference. At a dose of 30,000 r the binucleated germinating coccoids showed 48 per cent survivors and the binucleated bacillary cells only 2.7 per cent.

The culture was composed largely of coccoidal cells at  $28\frac{1}{2}$  hr but was more sensitive than the normal resting coccoids. This was probably due to the presence of some bacillary cells. At 36 hr the X-ray dose-survival response was essentially the same as that of the 5-day-old coccoids, and the cytological appearance was much the same (figure 43).

The increase in X-radiation resistance during germination is evidence against a reduction division at this stage. A cell containing 2 haploid nuclei should be considerably more sensitive than a similar cell containing a single diploid nucleus. The former has a hit multiplicity of 2 (Atwood and Norman, 1949) and the latter a hit multiplicity considerably greater (Tobias, 1952). Thus the hyphal nuclei appear to remain diploid until the fragmentation process begins. The radiation studies provided evidence that the nuclear division initiating fragmentation is reductional. Partial reduction division may explain the increase in sensitivity at 12 hr of the hyphae with discrete condensed nuclei. The X-ray sensitivity

of the binucleated bacillary stage may be explained by the presence of haploid "3 spot" nuclei. This dose-survival curve is very close to the 2 event type and thus corresponds to the 2 haploid nuclei (figure 34, curve 5).

The increased radiation resistance of the culture paralleled precisely the change in the composition of the culture from binucleated bacillary cells to uninucleated coccoidal cells. The evidence is strongly suggestive that nuclei within the bacillary cell fuse to form a uninucleated coccoidal cell. An exchange of nuclei may occur between adjacent bacillary cells through hyphal fusion and fusion tubes prior to nuclear fusion.

The integration of cytology and radiobiology into the cytogenetic approach provided evidence for the following sequence of events. Resting diploid coccoids germinate, forming diploid multinucleate coenocytic hyphae. A nuclear division, cytologically suggestive of a reduction division initiates hyphal fragmentation and gives rise to binucleated haploid bacillary cells. Two nuclei of the same or of adjacent bacillary cells fuse and form a single diploid nucleus typical of the coccoidal stage.

*Cultural characteristics and environmental effects.*

(1) Growth cycle variation.—Cultural conditions were found to greatly influence the morphology and duration of phases of the growth cycle. The influence of various carbohydrates in reducing the hyphal stage is shown in table 1. The data presented in this table were obtained using a standardized inoculum, since fragmentation is delayed and hyphae are much longer if very small inocula are used. Broth cultures revealed significant differences in fragmentation time depending on inoculum size. Fragmentation in fructose broth varied from 12 hr if a very large inoculum was used to 48 hr if a very small inoculum was used. The amount of aeration was also important in liquid cultures and the fragmentation process was much delayed or absent under reduced oxygen tension. The addition of fructose or glucose to the medium reduced the hyphal stage, both by shortening the average length of the hyphae and by decreasing the duration of the stage. Since the total volume of growth was increased upon the addition of glucose or fructose, the increased growth must occur in the coccoidal stage. Glucose or fructose concentrations above 2 per cent in nutrient broth were found to be inhibitory. The optimum concentration of glucose or fruc-



TABLE 1

*The effect of medium on the amount of growth, time of fragmentation and pigment intensity*

Medium	Maximum Average Hyphal Length	Time of Fragmentation	Relative Volume Growth	Involution Forms	Pigmentation Intensity
	$\mu$	hr			
Nutrient agar.....	15	14	++	—	++
Glucose agar.....	9	9	+++	—	+++
Fructose agar.....	8	8	++++	—	+++
Glycerol agar.....	12	14	++	++	++++
MacCloed's med. broth: aerated.....	15	20	+	+	+
Nutrient broth: aerated.....	30	30	++	—	+
Glucose broth: aerated.....	20	20	+++	—	+++
Fructose broth: aerated.....	20	18	++++	—	+++

tose in respect to total growth was about 0.75 per cent.

The addition of glycerol to the medium greatly increased pigment production and fat formation. The fat soluble, water insoluble pigment is located principally in the fat inclusions. The presence of glycerol in the medium resulted in decreased total growth and the stimulation of various types of involution forms.

(2) Colonial morphology and growth:—Surface colonial morphology of the normal stain of *N. corallina* on nutrient agar is typically rough and the ridged appearance shows little variation on various solid media. The moisture content of the medium was found to influence colonial morphology to a significant extent.

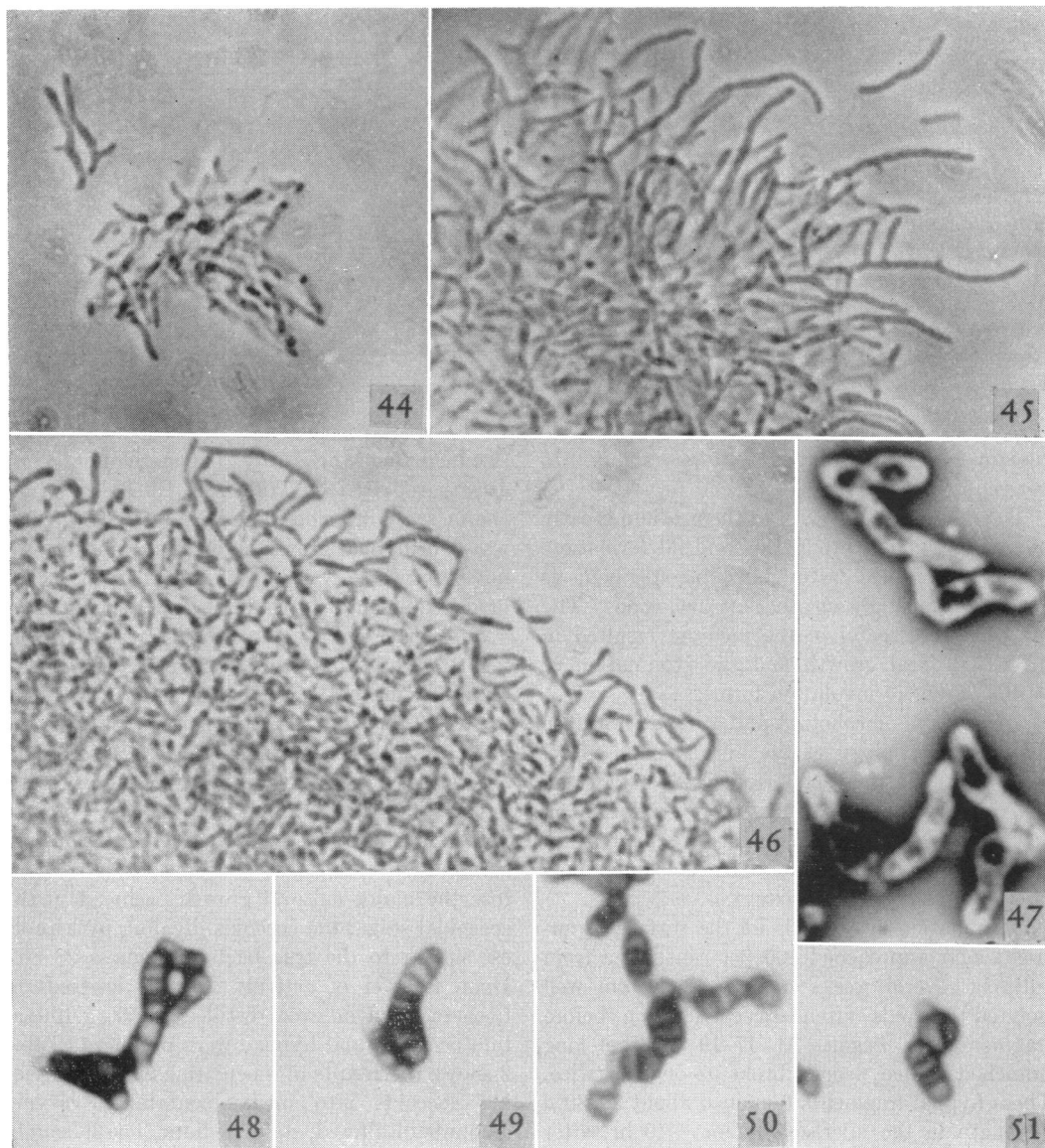
Germination of coccoids on the surface of nutrient agar approached 100 per cent, even from cultures several weeks old. Hyphae from well isolated coccoids often exceeded 50  $\mu$  before fragmentation began. At 17–19 hr the long branched hyphae usually broke up once or twice. These hyphal fragments remained about 20–30  $\mu$  in length in the microcolony for 8–10 hr, after which the length gradually shortened until the center of the colony was completely coccoidal at about 40 hr incubation at 28 C. Phase studies of microcolonies of various ages are shown in figures 44–46. The behavior of a single, well isolated coccoid during colony formation differed from that of a group of coccoids. The colony expanded by hyphal growth at the periphery for 50–55 hr after which most growth, both in thickness and in circumference, occurred by coccoidal division. Long hyphae continued to grow out from the margin. These hyphae were followed by a margin of dividing coccoids which produced most of the

size increase of the colony. The method of colony growth reported by Waksman (1950), in which the increase in colony size was accomplished by waves of hyphal growth and fragmentation, was not observed in *N. corallina* except in the 25–36 hr developmental phase of the microcolony.

Subsurface colonial growth differed from surface growth in that growth was much slower and remained in the hyphal stage until the colony was 8–10 days old. The hyphae remained in the 8–15  $\mu$  length range, undergoing single divisions as the length of individual hyphae increased beyond this range.

(3) Effect of substrate on fragmentation and germination:—On nutrient agar containing fructose the major cultural growth occurred in the coccoidal stage; the coccoids dividing by a process similar to the true bacteria (figures 28–30). Upon transfer of actively dividing coccoids to fresh medium or even distilled water, germination occurred and hyphae were produced. Table 2 shows the results of inoculating actively dividing coccoids into media containing varying amounts of filtered, stale medium. The inoculum size was constant in these tests. The stale medium caused a reduction in both hyphal growth and time of fragmentation. The most simple explanation for this phenomenon is the production of some metabolic product which initiates fragmentation when a certain critical concentration is reached.

Two parts stale medium to 1 part fresh medium completely inhibited coccoid germination. Since coccoids were found to germinate in distilled water this failure to germinate in stale medium is evidence for a germination inhibition factor. The fragmentation and germination inhibition



*Figures 44-51*

*Figure 44.* Phase study of 15 hr microcolony growing on nutrient agar. 1,000  $\times$ .

*Figure 45.* Phase study of 24 hr microcolony growing on nutrient agar. 1,000  $\times$ .

*Figure 46.* Phase study of 40 hr microcolony growing on nutrient agar. 1,000  $\times$ .

*Figure 47.* Crystal violet stain of large forms after 20 hr at 37 C. 3,000  $\times$ .

*Figures 48-49.* Cell wall stain of large multicellular forms after 25 hr at 37 C. 3,000  $\times$ .

*Figures 50-51.* Cell wall stain of large multicellular forms after 30 hr at 37 C. 3,000  $\times$ .

factors may be the same or different metabolic products. Apparently a metabolic product which builds up in the medium and hyphae initiates fragmentation and inhibits the germination of the resultant coccoids. However, it permits the coc-

coids to continue dividing under appropriate conditions.

There is evidence that the material accumulates inside the hyphae, since rapid repeated transfer of hyphal growth delayed, but did not

TABLE 2

The effect of the ratio of stale to fresh medium on germination, hyphal length and time of fragmentation

Stale Medium/ Fresh Medium Ratio	Hyphal Length	Time of Frag- mentation	Germination
	$\mu$	hr	
1/100	10-20	18-20	+
1/50	12-16	18-20	+
1/10	10-12	12-15	+
1/5	8-10	10-12	+
1/3	6-8	8-10	+
1/2	4-6	6-8	+
1/1	2-4	4-6	+
2/1*	—	—	—

\* Germination completely inhibited.

entirely prevent fragmentation. Upon transfer to fresh solid medium at 8 hr intervals the hyphae reached a maximum average length of about 12  $\mu$  for the first 6 transfers then shortened to a length of 6-10  $\mu$ , apparently dividing as the latter length was exceeded. Transferring the culture just after fragmentation had begun, resulted in the formation of some involution forms, but usually fragmentation, once initiated, continued to completion.

No attempt was made to isolate a "fragmentation factor" from the stale medium.

(4) Temperature effects:—Although the optimum temperature for maximum growth of *N. corallina* is 29-31 C, growth can occur at temperatures from 5-45 C. Incubation temperatures above 35 C produced extreme alterations in the growth cycle. At 37 C involution forms developed slowly from germinating coccoids (figure 47), and the fragmentation process which occurred at 20-24 hr produced extensive cross septation in the involution forms (figures 48 and 49). The polycellular units later fragmented forming a culture of large polycellular bacillary cells and coccoids (figures 50 and 51).

#### SUMMARY

Cytogenetic procedures, applicable to micro-organisms, were selected and tested on a suitable organism as a basis for the valid application of these procedures to other microorganisms. *Nocardia corallina* was chosen as a test organism on the basis of preliminary cytological studies.

The crystal violet nuclear stain, the thionine-

SO<sub>2</sub> nuclear stain, the crystal violet-tannic acid-congo red cell wall stain, and phase microscopy, were found to be valid tools of microbial cytology if interpreted with restraint.

The correlation of cytological and radiobiological findings indicates that, in *N. corallina*, diploid coccoidal stage gives rise to a coenocytic diploid hyphal stage which fragments through a nuclear reduction division to form haploid binucleated bacillary cells. The bacillary cell nuclei fuse and the cell divides to form diploid coccoids. The haploid "chromosome" number is suggested as 3 for this organism.

It has been demonstrated that a microbial cytogenetic approach involving the correlation and integration of cytological procedures with genetic and radiobiological methods can aid in solving basic problems of microbial cytology and genetics.

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