THE MORPHOLOGY AND CYTOLOGY OF MYXOCOCCUS XANTHUS, N. SP.[1]

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Knaysi (1938) states that there are three current points of view relative to the presence and nature of the bacterial nucleus. One group of investigators holds that the bacterial cell contains no nucleus. Knaysi argues, however, that the fact that a nucleus has not been seen is no indication that it does not exist. A second group maintains that nuclear material is present but in a highly dispersed condition and is therefore not easily seen. The third group takes the stand that the presence of a nucleus is logical, and is indicated by granular bodies often noted within the bacterial cell. These bodies are frequently observed to behave both functionally and chemically as nuclei are supposed to behave. In general, this latter view, that a more or less definite nucleus does exist, at least during certain stages of the life cycle of the bacterial cell, seems to be favored by many investigators today.

Stoughton (1929) observed the presence of stainable bodies in the cells of *Bacterium malvacearum*. Just before cell division occurred these bodies split, the halves migrating toward opposite ends of the cell, and finally were included in the new daughter cells. The dye reactions of these intracellular bodies were typically nuclear, and the author concluded that they should be considered as nuclei.

Much of the work published by the Hollandes (1930, 1932) favors the theory of a compact nucleus. Lindegren and Mellon (1932) described a type of reduction-division that took place

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within bacterial cells during certain phases of the life cycle. Badian (1935) stated that the condensed nuclear material to be seen in *Bacillus megatherium* may be observed to divide and rearrange itself in a manner quite suggestive of autogamy. Brooke (1936) studied a number of pathogenic bacteria and from his observations concluded that the granules he noted were composed of nuclear material. Allen, Appleby and Wolf (1937), working with a spore-forming bacillus, noted deep-staining bodies within the spores as well as within the vegetative cells. These bodies went through a rearrangement process after spore formation had taken place, forming recognizable structures of various shapes. The authors contended that the spore has some function other than merely acting as a resting stage during unfavorable periods of growth. Rosca (1937) described a type of nuclear activity that he considered asexual in certain cases, sexual in others. Although he did not actually observe the conjugation of two cells he could distinguish two types of chromosomes and considered them to be ♂ and ♀.

Chance's (1938) observations on *Bacillus mesentericus* disclosed a reorganization and redistribution of chromatin comparable to a mitotic division in higher forms, and Allen, Appleby and Wolf (1939) gave evidence of meiosis having taken place in a species of *Bacillus* which they studied. These same investigators also witnessed and accounted for at least two methods of spore formation in the one organism, and methods of reproduction other than the usual transverse fission. Lewis (1940) concluded that the theory of a more or less condensed nucleus, or at least a chromatin-incrusted gene string, is preferable to the theory of a diffuse nucleus. This worker pointed out that hypotheses of diffuse nuclei are not in keeping with findings on the transmission of hereditary characteristics.

Many of the publications on this subject deal with observations on spore-forming organisms. Possibly this is true because such organisms appear to undergo more marked cell reorganization just prior to, and during, spore formation. To the author's knowledge only two papers dealing with the cytology of myxobacteria have appeared. Krzemieniewski (1928) studying cells

of *Sorangium sorediatum*, *Polyangium fuscum* and of species of *Myxococcus*, *Archangium*, etc., and Badian (1930) working with *Myxococcus virescens* observed deeply-stained granules within the cells. These structures underwent a type of mitosis during the vegetative period of growth, and just before, and during, the process of sporulation displayed great variety of forms. Badian observed these intracellular structures to double in number, and then decrease by means of chromatin rearrangements. This was interpreted as a reduction-division, the whole process being considered a type of autogamy.

The present study began with the isolation and purification of a previously undescribed species of *Myxococcus*. In a number of ways this organism appears to be closely related to *Myxococcus virescens* (the species studied by Badian), particularly in the size of the vegetative cells and spores. These are larger than those of most of the other species of this group that have been observed in this laboratory. Because of the large size of the cells and the remarkable intracellular figures (one of the first things to be noticed), the species seemed to be well-adapted for cytological study. The species has been separated from *M. virescens* on the basis of the pigmentation of the fruiting body, a characteristic often used in the differentiation of species of *Myxobacteriales*. The name *Myxococcus xanthus* is proposed for this organism. The diagnosis is as follows:

MYXOCOCCUS XANTHUS

Etymology: Greek (adj.) = orange, golden.

Fruiting body: Spherical to subspherical, usually sessile but occasionally constricted at the base giving the appearance of a short stalk or foot. Mature fruiting body 300 to 400μ in diameter, often slightly flattened on top or on one side. Color varies from light yellowish-orange when young to bright orange when mature; color constant, never tending toward greenish yellow. No outer cyst wall or membrane discernible, the spores being imbedded in the slime holding the mass together. Usually single, though two or three fruiting bodies may become joined to form an irregular mass; each is attached to the substrate, however, and they never bud, one from another.

Spores (Resting cells): Spherical, with thick outer wall or membrane.

highly refractile when unstained. Stain very easily with any of the ordinary bacterial or nuclear dyes. 2.0μ in diameter, sometimes slightly larger.

Vegetative colony (Pseudoplasmodium, swarm): The characteristics vary with the substrate. On plain 1.5 per cent agar (no nutrients added): Very thin and transparent, often hardly visible except by reflected light. Little or no pigmentation. Surface covered with fine, more or less regularly spaced, ridges, causing a dull macroscopic appearance without gloss or sheen. Margin thin and quite irregular. On rabbit dung decoction agar: Colony thicker, the surface being broken by veins or ridges radiating from the center. Thick center area often smooth and glossy, while the margin appears much the same as that on plain agar. Veins or ridges extend outward in a loose spiral, always in a clockwise direction. Pigmentation, yellow to pale orange, is confined to the thicker central area, extends part way along the veins to the margin. On nutrient agar: Growth not good. Colony thick, at first heavily veined, the veins later merging to form an irregular glossy surface. Colony remains small, pigmentation usually fairly heavy; margin thick, irregular to lobate.

Vegetative cells: Large, flexible, single, gram-negative rods with rounded ends. No flagella, but move on the surface of a solid or semi-solid medium with a crawling or creeping motion. Vary in size from 0.5μ to 1.0μ by 4.0μ to 10.0μ; average 0.75 x 6.0μ. More or less distinct cell wall often evident.

Found growing on dry cow dung from a pasture near Ames, Iowa.

METHODS

The best growth of *M. xanthus*, as with all of the myxobacteria that have been studied in this laboratory, is to be had on a solid or semi-solid medium with some source of the more complex carbohydrates. The work of the Krzemieniewskis (1926) has shown rabbit dung to contain all of the necessary factors for most of the myxobacteria. Due to the methods used in making the slide preparations in the present study, rabbit dung did not lend itself particularly well to the process except in combination with other methods. While the rabbit dung plates described below were not developed with cytological work in mind, they proved to be satisfactory for the vegetative and spore stages of the life cycle. Rabbit dung decoction agar was employed for studying the germination of the spores.

Incubation was, for the most part, at room temperature. The work was carried on largely during the summer months and the laboratory temperatures of 22 to 27°C. proved to be satisfactory. Temperatures above 30°C. were not beneficial. At room temperature germination occurred in about 24 hours.

Culture methods

Rabbit dung plates were prepared by placing two or three pieces of rabbit dung in each petri dish and sterilizing, with the lids on, for one hour at 15 pounds pressure. When cool the plates were removed from the autoclave and allowed to stand over night in order to dry somewhat. Plain 1.5 per cent agar (no nutrients added) was then prepared. This was melted and sterilized in the autoclave for 20 minutes at 15 pounds pressure. When cooled to about 60°C. enough of the liquid agar was added to each plate to fill it to a depth of about one-half the diameter of the rabbit dung. This required 15 to 20 ml. of agar. Care was taken not to cover the dung with agar. Before the agar had a chance to harden, the pieces of dung were moved to the center of the plate with a flamed needle. When the agar had set the plates were inverted. Inoculations were made by transferring one or two fruiting bodies from the stock cultures to the dung at the level of the surface of the agar. Diffusion of the water-soluble parts of the dung into the surrounding agar made growth possible on the surface of the latter. Microscopic slide preparations were made from the colonies that developed on the agar around the imbedded pieces of dung.

Dung decoction agar was used in studying germination. This substrate was made by adding one liter of distilled water to 100 grams of dry dung. The mixture was heated to boiling and then allowed to infuse at room temperature for 24 hours. The solid material was then filtered off, and the filtrate made up to one liter by the addition of distilled water. Fifteen grams of agar were added and the solution sterilized in the autoclave for 30 minutes at 15 pounds pressure. When cooled to 60°C. plates were poured.

A fairly heavy suspension of spores was then made by transferring a few fruiting bodies to a sterile slide, inclosed in a sterile petri dish, on which a drop or two of sterile water had been placed.

The fruiting bodies were broken up with a flamed needle. Loopfuls of this suspension were then transferred to various points marked on the dung decoction agar plates. Incubation was at room temperature and slides were made at 12-, 24-, 48- and 72-hour intervals.

Methods of slide preparation

Originally an attempt was made to study the cells of *M. xanthus* by employing the usual type of smear technique. This was found to be entirely unsatisfactory. It was impossible to spread the material out on the slide, once it had been stripped from the agar substrate, due to the membranous nature of the colony. Those few cells that had been teased away from the mass were often so badly bent and distorted that the size and shape were questionable. Also, the fact that no indication of the disposition of the cells on the colony was to be had by this method made it imperative to use some other means.

Cover slip preparations were finally decided upon as being most satisfactory. For this purpose number one cover slips, seven-eighths inch square, were used. These were cleaned well and stored in alcohol. Just before use they were rinsed in 95 per cent alcohol and flamed. This was repeated twice. After the final flaming the cover slip was placed, by means of sterile forceps, over that section of the colony to be studied, and pressed down carefully to assure good contact. The only difficulty was with air bubbles, and it was found that with a little care these could be pressed out from under the glass. Generally the cover slips were left in place for about two minutes, though the time factor was not critical. The cover slips were then removed with sterile forceps, care being taken to raise the glass, not to slide it. Part of the plan was to obtain a picture of cell distribution and any sliding motion would, of course, disturb the location of the bacteria.

When dried, the cover slips were placed in 95 per cent ethyl alcohol for fixation. A fixing time of three minutes was found to be satisfactory. Various mixtures of alcohol and xylol were also

tried as fixatives but no improvement could be noted over the alcohol alone. After fixation the slides were allowed to dry in the air.

Staining methods

Gentian violet-iodine. As developed, this method was a modification of the gram technique. The fixed preparations were stained for five minutes in anilin oil-gentian violet, rinsed in distilled water, and then mordanted five minutes in Lugol's solution. This was followed by another wash in distilled water. The preparation was deeply stained, dark blue in color, with little differentiation between the various parts of the cell. A brief rinse in 50 per cent alcohol was enough to decolorize the non-chromatic parts of the cell and show up the nuclear structures. Since the cells are decidedly gram-negative decolorization was necessarily short. Counter staining was found to be superfluous and was dispensed with. Decolorization was stopped by a final wash in distilled water, and the preparations were dried in the air and mounted in neutral balsam.

Iron-hematoxylin. The fixed and dried preparations were mordanted in a 4.0 per cent solution of iron-alum for two hours, washed in distilled water, and placed in a well-ripened 1.0 per cent solution of hematoxylin (in 10.0 per cent alcohol) for two hours. This was followed by a rinse in distilled water. When differentiation was thought to be necessary a 1.0 per cent solution of iron-alum was used. Differentiation was less necessary in the case of this dye, however, than in the previous one, and for the most part was dispensed with. Counter staining was not employed.

Feulgen's stain. The Tomasi modification as described by Conn (1936) was followed. The fixed and dried preparations were given a brief rinse in cold 1/N HCl, placed in another 1/N HCl rinse (at 60°C.) for four minutes, and then rinsed again in cold 1/N HCl. This was followed by a wash in distilled water. They were then placed in a solution of decolorized basic fuchsin and allowed to stain for two hours. Decolorization was in an

acidified potassium metabisulphite solution. After rinsing in distilled water and drying, the cover slips were mounted in neutral balsam. Counter staining was not found to be practical.

The quality of the basic fuchsin used in this test is of prime importance. The first dye lot that was tried gave very poor results; another lot, certified for the particular purpose, was found to be satisfactory. Checks were run on slides of onion root tip in order to make sure that the procedure, as well as the dye, was correct before attempts were made to stain bacteria.

Other stains

A number of other dyes, for the most part the usual bacterial dyes, was tried in connection with this study. The majority of them were found to be unsatisfactory. Spore stains such as the Ziehl-Neilsen stain, the malachite green stain, etc., gave negative results on both spores and vegetative cells of *M. xanthus*. Both types of cells decolorized rapidly and completely. However, some of the differences between the spores of this organism and those of the true spore-forming bacteria were emphasized. Gram's stain gave negative results. Loeffler's methylene blue, while satisfactory in part, gave results inferior to those obtained with gentian violet and iron-hematoxylin. Safranin and carbol fuchsin were not satisfactory. Aceto-carmine and iron-acetocarmine were expected to give good reactions but rather poor results were had with both. Sudan III was tried in an attempt to show that the vacuoles occasionally seen within the cells were of fatty materials, but without success, and iodine in the form of Lugol's solution and as a dilute alcoholic solution was used as a test for glycogen. It gave negative results in the case of the vegetative cells, doubtfully positive in the case of the spores.

Forty-five millimeter petri dishes were employed for all procedures that required more than a few minutes for mordanting and staining. These were more satisfactory than larger containers since the small preparations could be processed separately

MORPHOLOGY AND CYTOLOGY

The germination of the spores, or resting cells, might be considered as the beginning of the life cycle of *M. xanthus*, but the

vegetative phase appears to be the dominant one; for that reason the vegetative phase is considered first. The vegetative cells

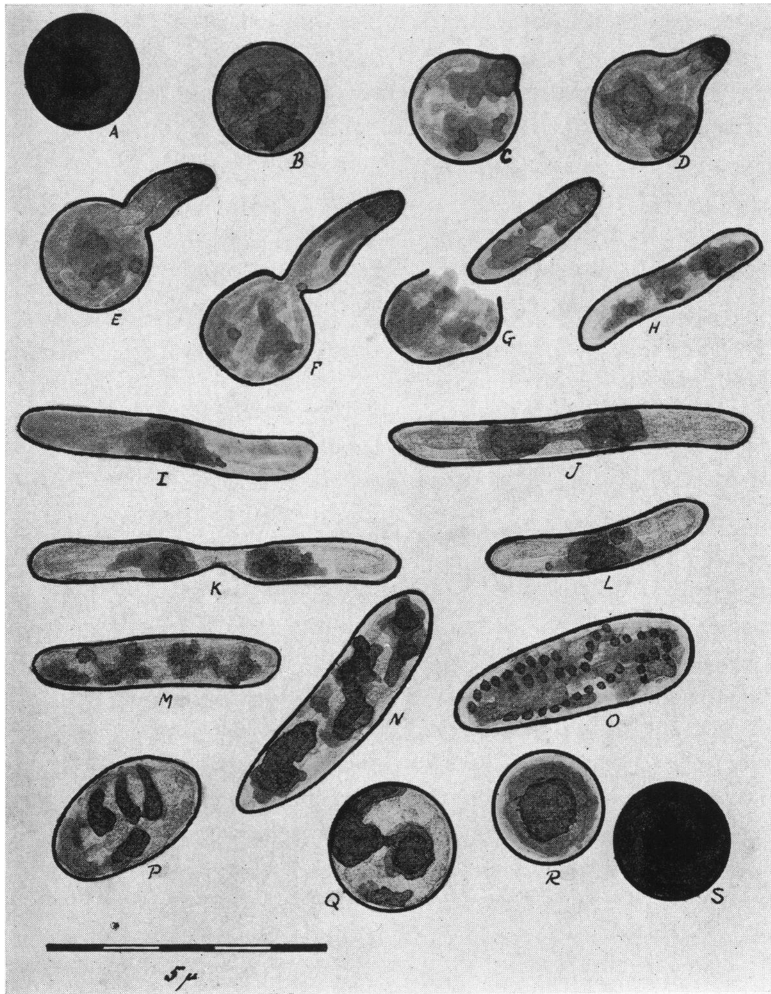

FIG. 1. RECONSTRUCTION OF THE LIFE CYCLE OF M. XANTHUS
Originally drawn to a scale: 1 cm. = 1 μ. A, mature spore; B, early germinal stage; C–H, germination; I–L, vegetative phases; M, transitional; N–O, prophase; P, early sporulation stage; Q–R, sporulation; S, mature spore.

of *Myxococcus xanthus* are long, flexible, gram-negative rods with rounded ends (Figs. 1 (I–L), 9 to 11). They have no flagella but appear to move by means of a crawling or creeping motion on top

of the layer of slime with which they pave the substrate. The exact nature of this motion is not well understood, but it has been suggested to be the result of an asymmetric excretion of slime by the cell, producing more pressure on one end than on the other. No wriggling motion such as is usually associated with motile bacteria can be observed, but the cell can be seen to change its position, from time to time, in relation to some fixed point. Examinations of living preparations of *M. xanthus* have shown the cells to travel at a rate of about 7μ per minute. The cells always move away from the center of the colony, but not in a straight line: there is always a curving of the path in a clockwise direction. This varies in intensity but may usually be noted to some degree.

The distribution of the cells on the colony as shown in figures 9 to 11 is characteristic not only of this particular species but of the genus. Very seldom is more than a single layer of cells observed on a colony. The cells are usually arranged in small groups of from two or three to a dozen or more, lined up with their long axes parallel, and moving in the same direction. The entire group moves as a unit, along a "front." While a few of the cells seem to be traveling independently, the large majority always follow the same slightly curved path toward the margin of the colony. Generally they are not packed closely together, but are separated by about the diameter of one cell.

The vegetative cells average 6.0μ in length by about 0.75μ in diameter. Unstained, the cells are long flexible rods, usually rounded on the ends. However, they occasionally appear to be slightly tapered at one or both ends, particularly those cells at the margin of the colony; very few are spindle-shaped. More often than not the younger unstained cells show several highly refractile granular bodies, varying in number from two to eight, while the older vegetative cells show one or two rather large refractile bodies at or near the center of the cell. The fixing and staining procedures previously outlined appear to have very little effect on cell morphology, the only noticeable change being an extremely slight swelling at the ends of the younger cells. This gives them the cylindrical appearance of the older cells.

The cells stain fairly well with most of the usual bacterial dyes, in some cases a thin cell wall being visible. The center of the cell is occupied by a deeply-staining body approximately one-third the length of the cell and equal in diameter to the cell; i.e., about 2.0 by 0.75μ. As far as can be told at the present time this body has no limiting wall or membrane, but is rather a mass of compact nuclear material. It has a marked affinity for gentian violet and for iron-hematoxylin, and gives a positive reaction with Feulgen's stain. The remaining portions of the cell take all stains lightly. Occasionally the cell appears to contain vacuoles at one or both ends but tests for glycogen and for fats have failed to indicate the nature of the vacuolar material. Functionally the condensed body located at the center of the cell seems to be nuclear, also. Vegetative reproduction is by means of transverse fission. Prior to cell fission a division of the nucleus takes place. Stages in this process may be seen in figures 1 (I–L), 9 to 11. At first the nucleus enlarges longitudinally to almost twice its length and then begins to constrict at a point near its center, producing a dumbbell-shaped body. The halves finally pull apart and migrate toward opposite ends of the cell. After nuclear division has been completed actual cell fission begins. This also is accomplished by constriction and not by the formation of a transverse wall. The steps in the process are shown in figures 1 (I–L) and 9 to 11. No case has been observed in which the daughter cells remain united in pairs after cell division has been completed. Each new cell contains the single, deep-staining, compact nucleus. Sometimes, during periods of rapid growth, a second nuclear division takes place before fission begins, and in such cases cells with four nuclei may be noted. These are not to be confused with a later stage in which four stainable bodies are typical.

The vegetative phase continues for 4 to 10 days, the time depending upon conditions of temperature and environment. At room temperature (21 to 25°C.) and on rabbit dung plates the first signs of fruiting body formation begin to be noticed after five to six days. Morphologically, a slight increase in the diameter of the cells is the first indication of the change about to occur

(fig. 1 (M), 12). Most of the cells measure approximately 1.0μ in diameter in this early transitional phase, and this increase in diameter may also be accompanied by a slight decrease in length. At first there is not much change in the internal structure of the cell, but soon the nucleus is noted to have broken up, and particles of deep-staining material are to be seen throughout the length of the cell (figs. 1 (M), 12 to 16). During this stage the cells begin their migrations toward some predetermined "fruiting center" on the colony.

The stimulus for the gathering of the cells from within a given area for the express purpose of sporulation and the formation of fruiting bodies is not understood. No apparent change in temperature or environment is necessary although a lack of food material may play a part. More likely would be the production of metabolic products by the cells, or a slight change in the moisture content of the medium. At any rate, the motion of the cells toward the margin of the colony suddenly ceases, and all cells within a given area begin to move toward a central point. This is best shown in figure 17. As the bacteria approach this point, the morphological and cytological changes taking place are particularly marked. Observations by several workers on a species of *Bacillus*, and by Krzemieniewski (1928) and Badian (1930) on species of myxobacteria, have indicated that a number of changes occur within the cell prior to sporulation; whether, as in the case of species of the *Bacillaceae*, the spore is formed as a structure within the cell, or whether, as in the case of *Myxococcus xanthus* and other myxobacteria, the cell is the spore, rearrangement and redistribution of chromatin material appears to be a preliminary step in sporulation.

As they approach the location where the fruiting body is to be formed, or is in the process of formation, the cells increase in diameter and become perceptably shorter (figs. 1 (N), 3, 14, 15, 16), and rearrangements in the chromatin material become quite apparent. The chromatin previously distributed throughout the length of the cell collects in four more or less distinct masses (figs. 12 to 16). These bodies are not arranged within the cell in any particular fashion in the earlier stages, nor do they

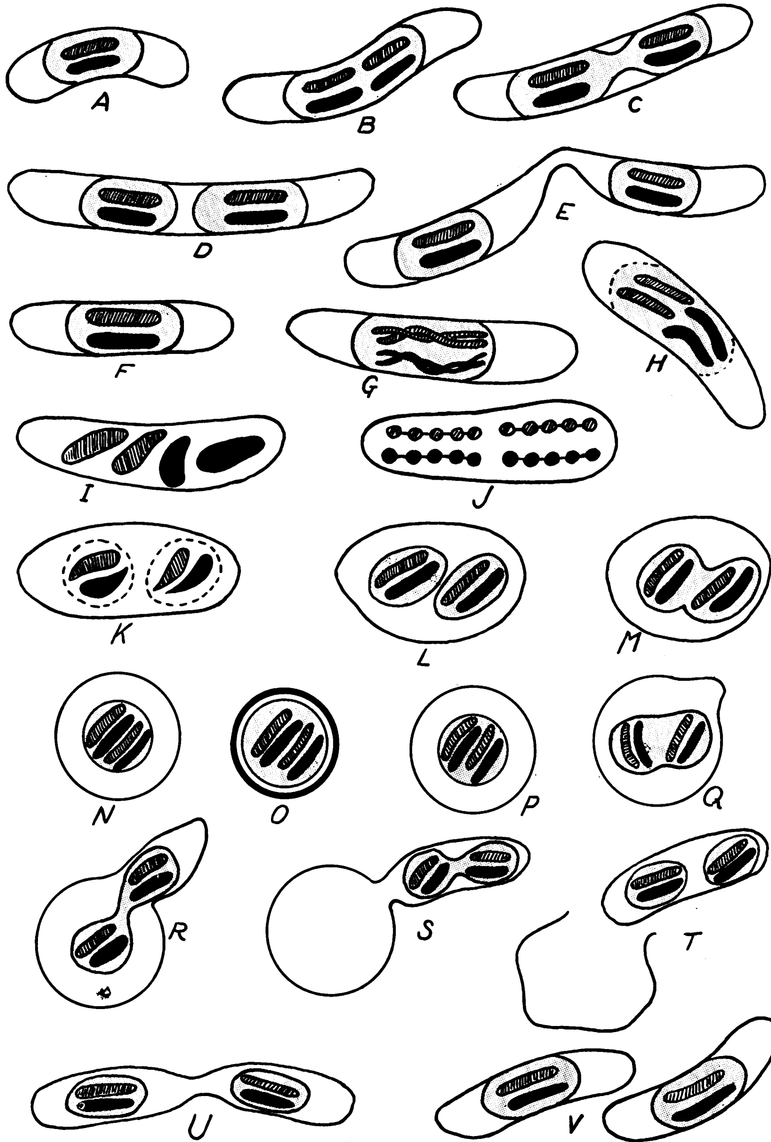

FIG. 2. DIAGRAMMATIC OUTLINE OF NUCLEAR DIVISIONS; NOT DRAWN TO SCALE

A–F, vegetative phases; G–J, prophase; K, late prophase; L, chromosomal fusion; M, nuclear fusion; N–O, spore, mononucleate, diploid; P–Q, early germinal, diploid; R–S, germination; T, binucleate vegetative; U, post-germinal division; V, haploid vegetative cells.

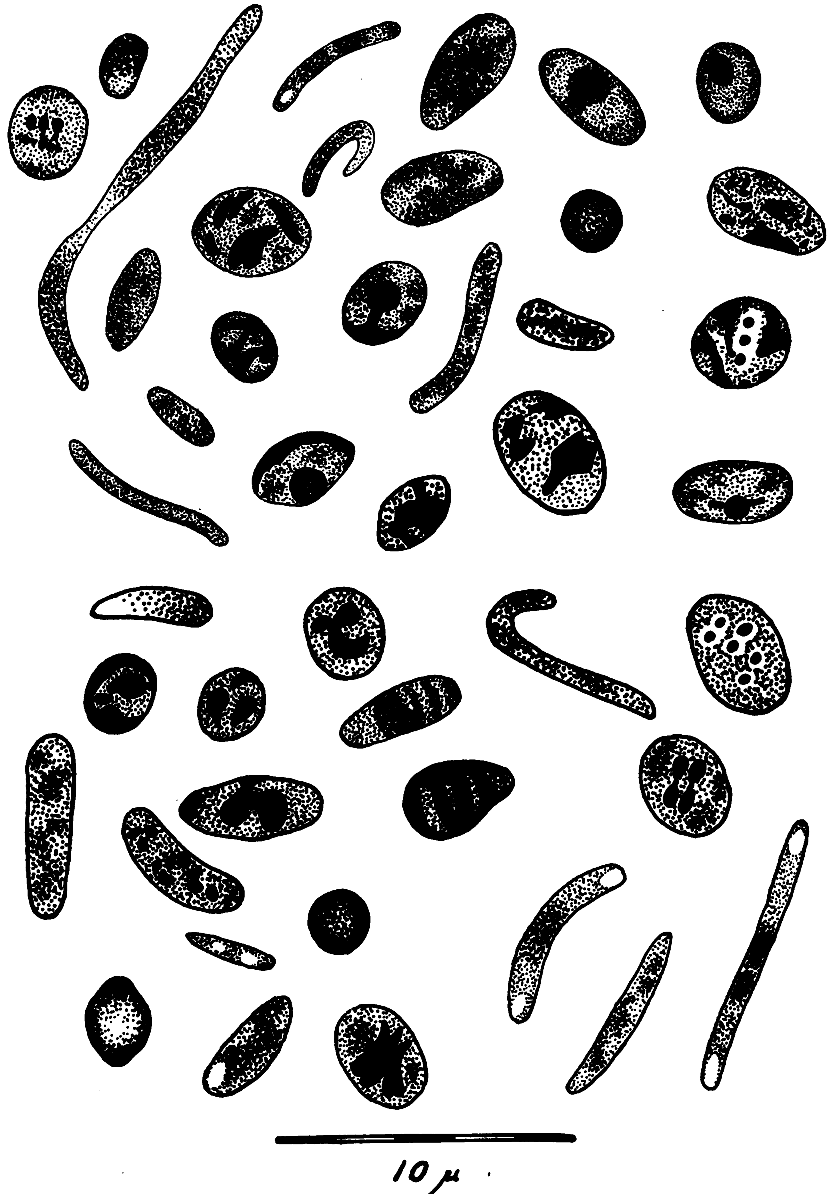

FIG. 3. VARIOUS STAGES IN THE LIFE CYCLE (EXCLUDING GERMINATION) OF M. XANTHUS

Drawn to scale: 1 cm. = 2 μ. Figures from preparations stained with iron-hematoxylin and gentian violet-iodine. Measurements by means of a Whipple eye piece.

seem to have any particular shape although their outlines are sharp. In general, they give the cell a banded appearance; two of them are usually located at the poles, the other two near the center of the cell. At this stage the cell is about 1.0 to 1.5μ in diameter by 4.0 to 5.0μ in length. There is a good deal of variation in cell size during these phases, and cells as broad as 2.0μ have been noted.

The next phase is typified by an elongation of the four structures into rod-shaped bodies or chromosomes. These become arranged in pairs with their long axes parallel to each other and to the long axis of the cell. One pair tends to locate toward each end of the cell. These rod-shaped chromosomes then begin to break up into chains of bead-like bodies that are interpreted as chromomeres. The break is not complete, however, for the chromomeres remain in a chain, and under proper lighting can be seen to be held together by slender threads of chromatin material. There is little doubt that these bodies are chromosomal in nature; they stain readily with the nuclear dyes, particularly iron-hematoxylin, and seem to function as prophase chromosomes in cells of higher forms. The chromomeres are refractile, and were so photographed. It will be noted in figure 17 that those cells nearer the margins of the illustration are in slightly different focus and show the chromomeres as deeply stained bodies within the cells. The number of chromomeres to each chromosome is difficult to determine, though total counts, i.e., the total number of chromomeres per cell, varied up to about 28. This would indicate a maximum of seven to each chromosome, if they were divided evenly between the four structures. In this stage the chromosomes are still typically in pairs, one toward each end of the cell. This stage appears to be of brief duration, having been observed only a few times during the examination of 350 to 400 slides, but it is thought to be typical. The illustrations, figures 17 and 18, were made from a slide in which nearly all of the cells were in this particular stage and afforded a good opportunity to study the structures. In all other cases only a few cells in the "prophase" stage were to be noted, and these more or less isolated from each other. In appearance, however, they were

all very similar. To this author's knowledge this is the first time any structures resembling prophase chromosomes have been observed in bacteria of any kind.

These long chain-like bodies soon shorten to oval, rod- or comma-shaped structures (figures 1 (P), 2 (K), 3, 23, 24, 25, 26) and appear to undergo a type of autogamous fusion. The cells, in many instances, are nearly spherical, often 2.5 to 3.0μ in diameter or larger, allowing the chromosomes to assume almost any position within the cell. In some cells they are seen to be paired, some of these being shown in figure 3, while in other cells only two large bodies are to be noted, indicating a fusion has occurred. This corresponds to the phenomenon described by Badian (1930) as taking place in *Myxococcus virescens* and considered by that author as being autogamous fusion.

During the fusion of the pairs of chromosomes, to form a binucleate cell, the size of the cells may be seen to begin to decrease (figs. 23, 24), and the staining reactions to become more marked. Often it is hard to differentiate the internal and external parts of the cell. The cell wall begins to thicken and stain more deeply (figs. 1 (Q), 20 to 27) and the two chromatic bodies, or nuclei, if not actually joined together, become closely appressed as though about to unite to form a cell with one large nucleus. It is thought that this actually does take place either during spore formation or while the cell is in the so-called resting stage. The latter assumption appears to be possible. Allen, Appleby and Wolf (1937) noted marked changes in nuclear structures in the spores of *Bacillus sp.* while the cell was supposed to be in a resting condition. Further indication of nuclear activity during the matured, or nearly matured, spore stage of *M. xanthus* is the fact that germinating cells frequently show a single large nucleus in the process of division.

The ripe spore is a spherical cell almost exactly 2.0μ in diameter. Unstained, it is highly refractile, and has a thick, easily visible wall. The spore is highly receptive to all bacterial and nuclear stains that have been tried, giving in most cases a very dense, completely opaque preparation with no internal structures visible. It is definitely positive to Feulgen's stain, the entire cell becoming

pink. This is not true of the previously described stages in which only the nuclear bodies gave a positive reaction. Gentian-violet-iodine gives a dark blue, almost black, color, and iron-hematoxylin colors the spore black with no details visible. Cells so stained are shown in figures 16 to 24. Gram's stain shows the spores, like the vegetative cells, to be markedly gram-negative, but when a somewhat specialized technique was employed, i.e., intense staining and mordanting and careful destaining, combined with dilute counter stain, one large gram-positive area within the gram-negative cell could often be seen. Whether this was due to incomplete destaining or not is uncertain. The fact that the gram-positive areas were not always centrally located might indicate some differences between their composition and that of the rest of the cell. If it were entirely a matter of incomplete destaining it would be reasonable to expect that the area would be centrally located since the entire cell wall is likely to be of equal permeability throughout. This would appear to support the assumption that the two nuclei in the immature spore combine to form a single large nucleus. By the time the cell reaches this stage it is imbedded in the slimy material holding the fruiting body together (fig. 5).

The differences between the spores of *M. xanthus* and those of species of *Bacillaceae* are notable. Spore stains such as Ziehl-Neilsen and the malachite green stains that show a definite differentiation between the spore and the sporangium in the case of the true bacteria show no difference whatever between the spores and the vegetative cells of *M. xanthus*. Decolorization is complete in both cases. On the other hand, stains such as Loeffler's methylene blue, gentian violet and the like, that ordinarily indicate the presence of a spore by a complete absence of color (with the ordinary techniques), produce a deep, opaque coloration in the spores of *M. xanthus*. Species of *Bacillus* generally form the spore within the cell, whereas in *M. xanthus* the spore is the cell, the entire structure of which takes part in sporulation.

Germination of the spores seems to occur only when a new environment is made available. It would hardly seem possible that the growth of a single layer of bacteria over the surface of

the substrate and the eventual production of fruiting bodies would exhaust the food supply of that area. It is more likely that the various metabolic products formed by the cell, or an unfavorable change, or lack of change, in moisture content, might cause the environment to become unsuitable for germination. Once fruiting has taken place little, if any, more vegetative growth is to be seen in that given area. Attempts to promote germina-

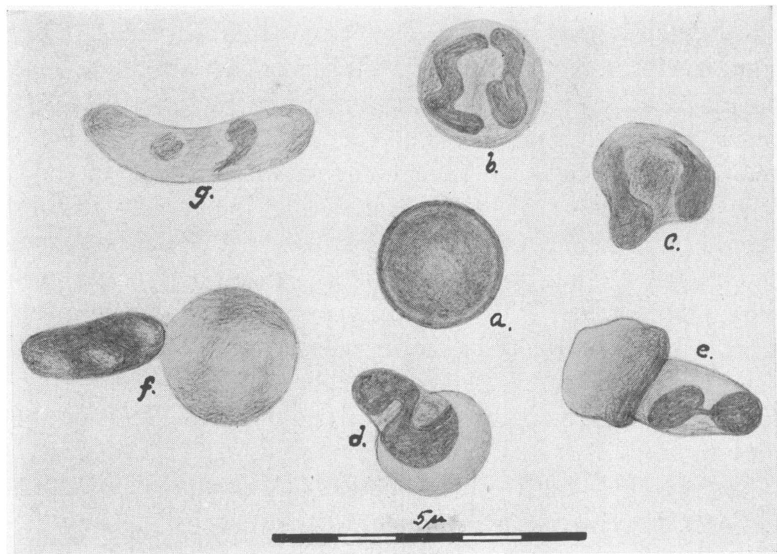

FIG. 4. STAGES IN THE GERMINATION OF M. XANTHUS

Drawn to scale: 1 cm. = 1 μ. A, spore; B, early stage in germination; division of nucleus has already occurred; C, first stage in formation of new cell: nucleus dividing; D, later stage; E, germination nearly complete: nucleus dividing; F, newly germinated cell and old spore wall; G, binucleate vegetative cell before first division.

tion on the same substrate on which fruiting took place failed. Transfer of some of the fruiting bodies to a fresh medium produces a vigorous new growth.

Studies on the germination of the spores were carried on by growing the cells on a dung decoction agar, rather than on sterilized rabbit dung imbedded in agar. Slide preparations made after 12 hours incubation at room temperature showed no signs of any changes having occurred in the spores. At the end of a

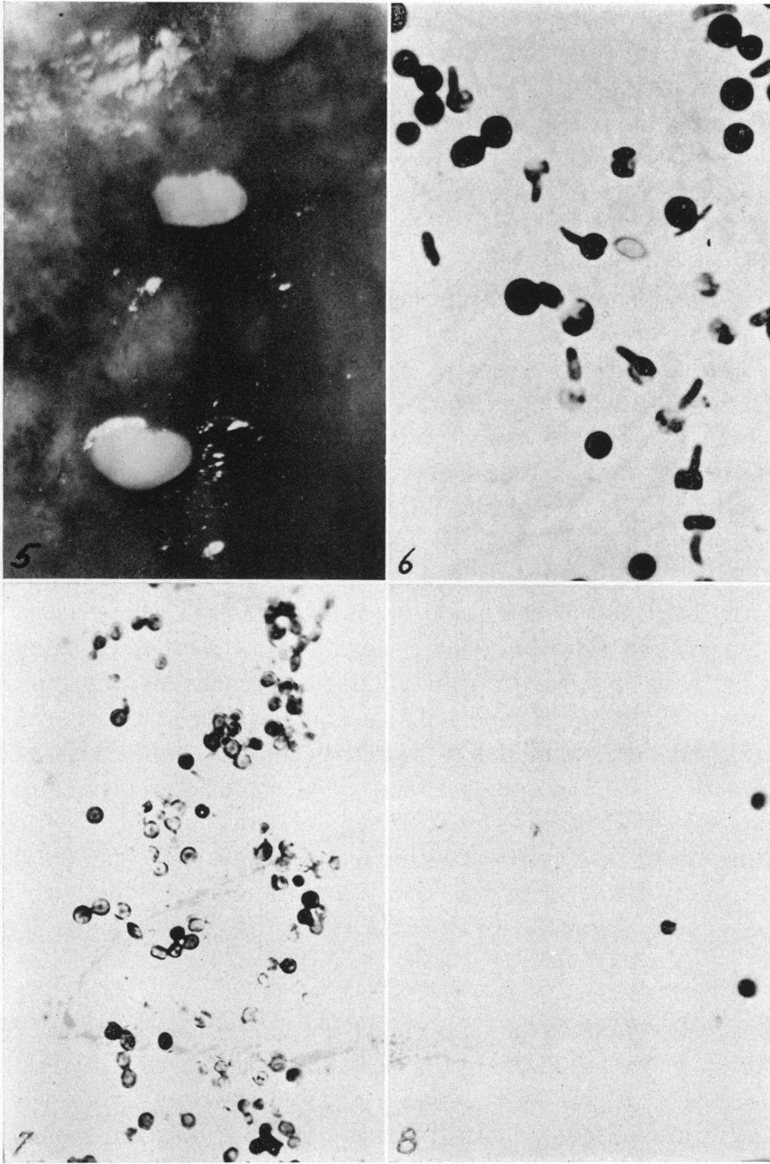

FIG. 5. Fruiting bodies of *M. xanthus* growing on rabbit dung. About 20 ×.
FIG. 6. Germinating cells in various stages. Gentian violet-iodine. 1860 ×.
FIG. 7. Same as No. 6. Iron-hematoxylin. 1160×.
FIG. 8. Same as No. 7. Newly formed binucleate cells may be seen.

24-hour incubation period at least half of the cells had begun the process of germination (fig. 6) while some of them had completed it.

The first indication of a change was the lessened affinity of the cell for dyes (figs. 1 (B), 6 to 8). During the earliest germinal phases, the internal structures of the cells are very difficult to observe (figs. 1 (A), 6 to 7) but, shortly, one or two stainable bodies are to be noted (figs. 1 (B), 4, 7, 8). There next appears a softening at some point on the cell wall, which has in general become much thinner, and a slight bulge appears. The stainable body, nucleus, migrates toward that point (figs. 1 (C), 4, 6, 7) and moves into the vegetative cell as the latter forms. Often the nucleus is to be seen as a dumbbell-shaped body during this stage (fig. 4, C, D, E) and may take a position at the distal end of the newly germinating cell and remain there until germination is complete (fig. 1, D–G). As a general thing nuclear division is complete by the time the cell has germinated, division being accomplished by constriction as in the case of the vegetative nucleus. The new binucleate vegetative cell (fig. 8) is completely freed of the spore wall when it has reached a length of 3.0 to 4.0μ. Until one division of this binucleate cell has occurred it can hardly be considered a typical vegetative cell. This post-germinal division seems to take place soon after germination, giving rise to two mononucleate vegetative cells. The new cells acquire the ability to move within a short time, and after 48 hours will usually have formed a colony large enough to see with the unaided eye.

DISCUSSION

The cells of *Myxococcus xanthus* go through a comparatively complex morphological cycle. In the vegetative (dominant) phase the cells are long, slender, flexible rods with rounded or slightly tapered ends. Multiplication is by transverse fission by means of constriction rather than by the formation of a transverse septum. The cells crawl over a layer of slime which they excrete on the substrate. Motion always follows a clockwise spiral path away from the center of the colony. The cells are often arranged in small groups which move as a unit.

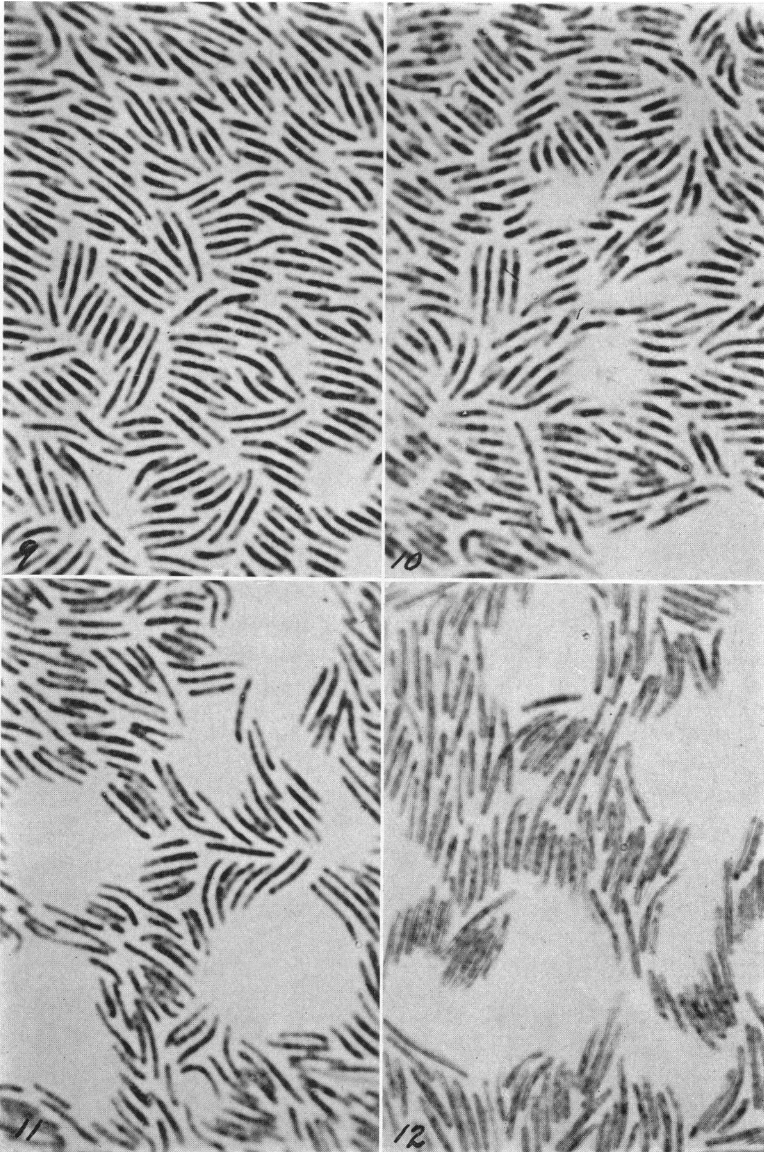

FIG. 9. Vegetative cells, showing typical distribution. Stages of vegetative reproduction may be seen. Gentian violet-iodine. 1860×.

FIG. 10. Same as No. 9.

FIG. 11. Late vegetative stage showing some cells with four deeply stained bodies. Gentian violet-iodine. 1860×.

FIG. 12. Late vegetative stage showing cells with nucleus breaking up into four bodies. Iron-hematoxylin. 1860×.

After several days in the vegetative phase the cells begin to gather around various fruiting centers. Changes in morphology are apparent, particularly a shortening of the cells. As they approach the point where the fruiting body is in process of formation the cells may become almost spherical. By the time they are incorporated in the slimy mass of the fruiting body the cell has become a perfectly spherical, thick-walled, non-motile spore.

Germination occurs when the spores are placed in a new environment. The first indication of germination is a thinning of the cell wall. At some point on the wall a slight bulge appears. This develops, by means of a process somewhat similar to budding, into an elongate rod-shaped cell. When this cell has reached a length of 3.0 to 4.0μ it becomes detached from the old spore wall by constriction at the point of emergence. After a single post-germinal division the cell is typically vegetative and begins the life cycle again.

Cytologically the life cycle of *M. xanthus* is correspondingly complex. In the vegetative phase a single large nucleus, occupying the central third of the cell, is to be seen. This body has a marked affinity for such nuclear stains as iron-hematoxylin and gentian violet, and gives a positive Feulgen reaction. There seems to be no limiting membrane inclosing the nucleus, but it is rather composed of a compact condensed mass of nuclear protoplasm. Its boundaries are quite definite. It seems to be granular in structure and apparently contains the equivalent of one pair of chromosomes (fig. 2 A). There is no evident arrangement in threads or chains such as might be found in the close spireme phase in the cells of higher plants. Nuclear division is by means of non-random amitosis. Prior to cell division the nucleus increases in size (fig. 2 B), along with the increase in size of the cell itself. Each particle of which the nucleus is composed must double in size and split, the various halves migrating toward opposite poles of the cell. This corresponds to the splitting of chromosomes before a straight somatic nuclear division: each new cell receives its portion of chromatin material carrying hereditary genes. After enlarging, the nucleus begins to constrict at a point near the middle (fig. 2 C), the halves pulling toward opposite

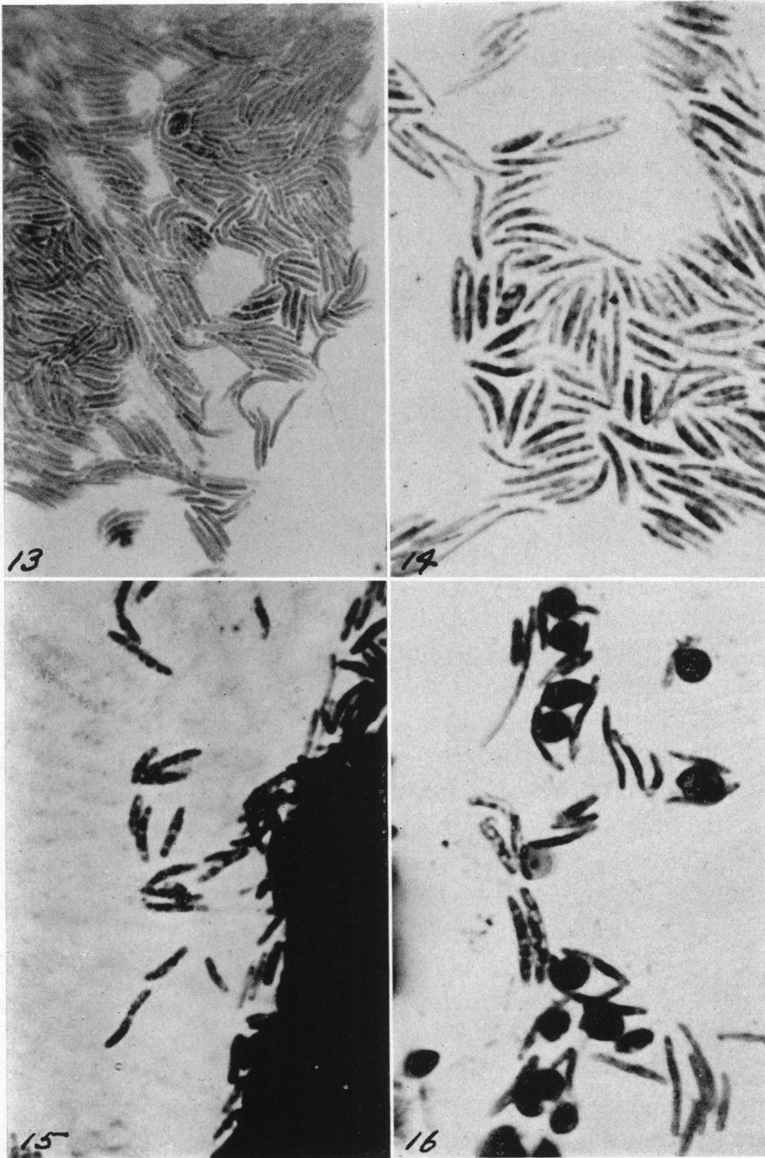

FIG. 13. Late vegetative and early transitional stages. Cells with two and four chromosomal bodies. Iron-hematoxylin. 1860×.

FIG. 14. Same.

FIG. 15. Same. Gentian violet-iodine. 1860×.

FIG. 16. Various stages in spore formation. Gentian violet-iodine. 1860×.

ends of the cell. Just before division has been accomplished, and before cell fission is obviously under way, the nucleus has the appearance of a dumbbell-shaped body (fig. 2 C). This condition has been at times previously described as the normal condition of the nucleus; actually it is a stage of comparatively brief duration. Nuclear division is always completed before cell fission begins (fig. 2 D); in a rapidly growing colony two nuclear divisions may take place before the cell itself has completed one division. Such cells appear to have four nuclei. This condition is not similar to a later stage in which four bodies, chromosomes, are to be seen within the cell. The two should not be confused. Once nuclear division is complete, the cell constricts at the middle (fig. 2 E), producing two new mononucleate daughter cells. This type of vegetative reproduction continues for an indefinite period of time.

When the cells begin to converge upon a fruiting center definite changes in cell structure begin to be seen. The first noticeable change is an increase, slight in some cases, in the size of the nucleus (fig. 2 F). The cell at this point is quite broad, and the nucleus, at first occupying a central position as in the typical vegetative phase, begins to spread out and become dispersed through the length of the cell; at this particular stage it might be considered diffuse (fig. 2 H). Shortly, however, four masses of nuclear material are to be noted, each corresponding to a chromosome (fig. 2 I). At first these are irregularly shaped masses, occupying various positions in the cell, but as the cell progresses toward the fruiting center the bodies within become more definite until four distinct structures may be seen. They elongate to rod-shaped bodies, pair up, and each chromosome breaks up into a chain of chromomeres (fig. 2 J). In this stage the chromosomes are similar, if not identical, to the prophase chromosomes in the cells of higher plants. Chemically and functionally these structures are chromosomal in nature. This stage is of brief duration, having been observed only a few times during the examination of numbers of slides, but it is thought to be a typical step in the mitotic division of the nucleus of *M. xanthus*.

Having become arranged in pairs the prophase chromosomes

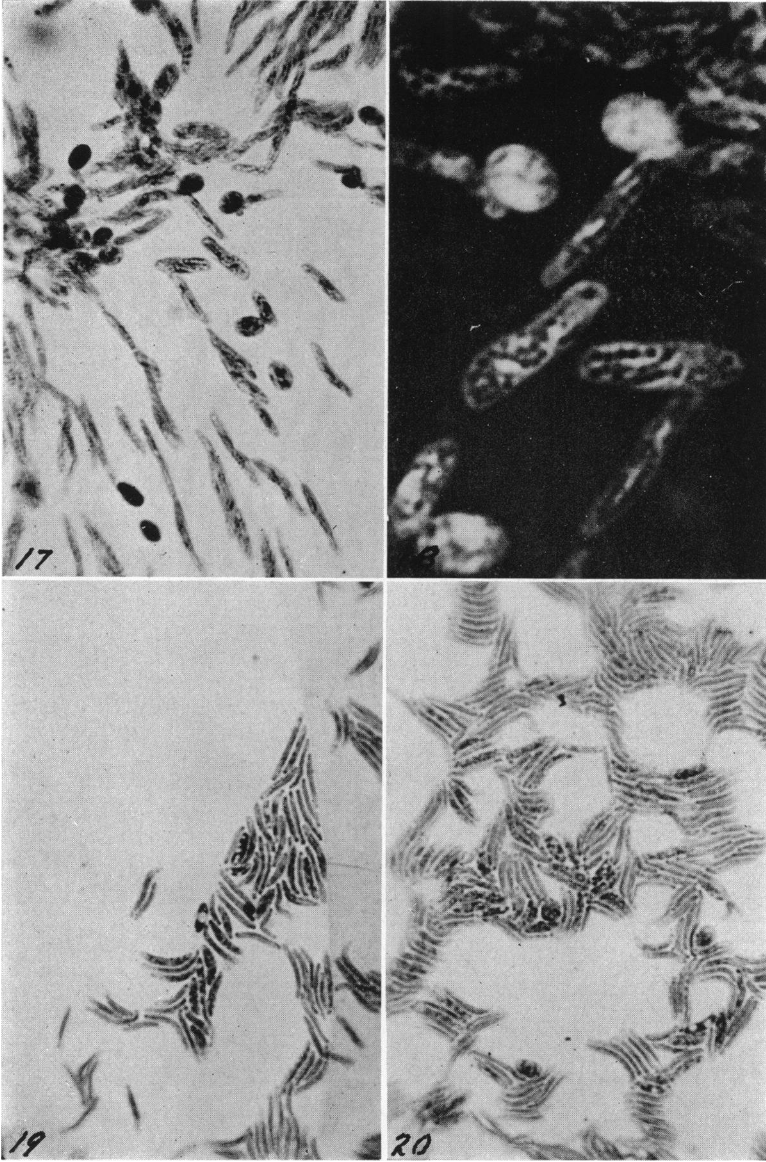

FIG. 17. Cells with chromosomes in the prophase stage. Migration of cells toward the fruiting center may be noted. Focus at the center is slightly different than at the margins of the illustration giving the chromatin material of the cells nearest a refractile appearance; cells near the margins show the chromosomes to be stained black with iron-hematoxylin. 1160×.

FIG. 18. Enlargement of cells from the center of No. 17. This illustration appears negative as the final print was made from a dia-positive rather than from an additional negative. Chromosomes are deeply stained but were focused to show as highly refractile bodies since they are more easily seen; they thus appear black in the negative print. 3500×.

FIG. 19. Various stages in sporulation. A few cells containing large vacuoles; otherwise nuclear material. Iron-hematoxylin. 1160×.

FIG. 20. Stages of sporulation. Iron-hematoxylin. 1860×.

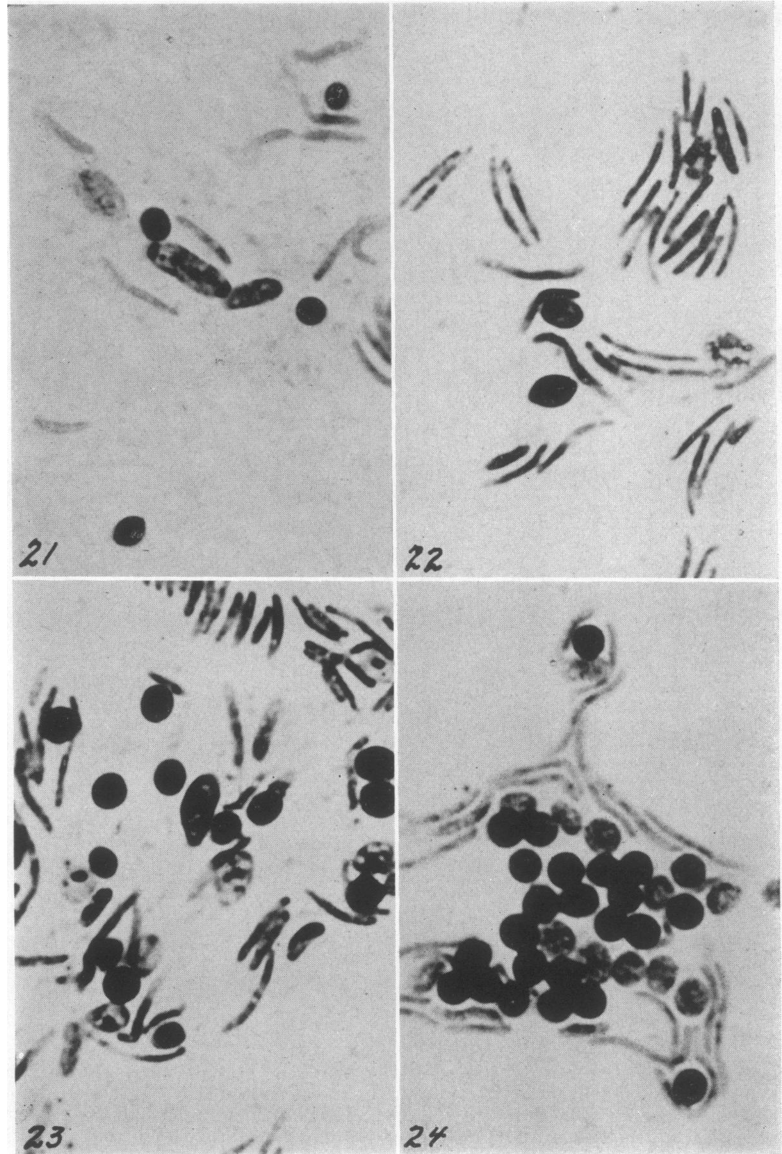

FIG. 21. Cells with "polar caps" of nuclear material. Not a typical chromatin arrangement. Iron-hematoxylin. 1860×.

FIG. 22. Stages in sporulation. One cell in oval stage, lightly stained; one showing two nuclei, joined together; one nearly matured spore. Gentian violet-iodine. 1860×.

FIG. 23. Cells with one-, two- and four-chromosomal bodies. Gentian violet-iodine. 1860×.

FIG. 24. Late stage in spore formation. Iron-hematoxylin. 1860×.

begin to shorten, becoming rod-, L- or comma-shaped (fig. 2, K). The cells are much thickened and shortened by this time, and the arrangement of these pairs of chromosomes varies considerably. No correlation to the long axis of the cell has been noted. Finally a union of the pairs of chromosomes is accomplished, producing the binucleate cell (fig. 2, L), each nucleus containing the equivalent of one pair of chromosomes. As a rule the two nuclei are about the same size, although in some cases one may appear to be somewhat larger than the other. Rosca (1937) based his theory of sexual union on the pairing of two bodies unlike in size. It is thought that any difference in the size of these two nuclei in *M. xanthus* is purely incidental.

At this point the cell has become almost spherical, and the cell wall has begun to thicken. The spore is already imbedded in the fruiting body and must have lost all power of locomotion. No motile spores have ever been seen. It is quite probable that a second nuclear union takes place at this time or after the spore has been formed. Due to the extreme affinity of the cell for dyes and the thickness of the cell wall it has been found impossible to observe changes in the nuclear structure during these phases. However, the division of a single large nucleus in the early stages of germination points toward a previous autogamous nuclear fusion (fig. 2, M–N) and it is thought that the spore goes through the resting stage as a large single nucleus, diploid in nature, surrounded by a thick cell wall, and containing little, if any, other cytoplasmic material. That the spore is basically nuclear is shown by the dye reactions, particularly the definitely positive Feulgen reaction. No vacuoles of any sort have been noted within the spores, and the test for glycogen was questionably positive. Apparently little reserve food material is present. An occasional pre-spore stage cell may show one or two vacuoles, the remainder of the cell giving positive nuclear reactions. These cells are not often seen, however, and attempts to ascertain the nature of the vacuolar material were unsuccessful.

Germination of the spores is first indicated by a lessened affinity for dyes. At this time the large single nucleus initiates a pregerminal division (fig. 2, Q) that progresses as the spore germi-

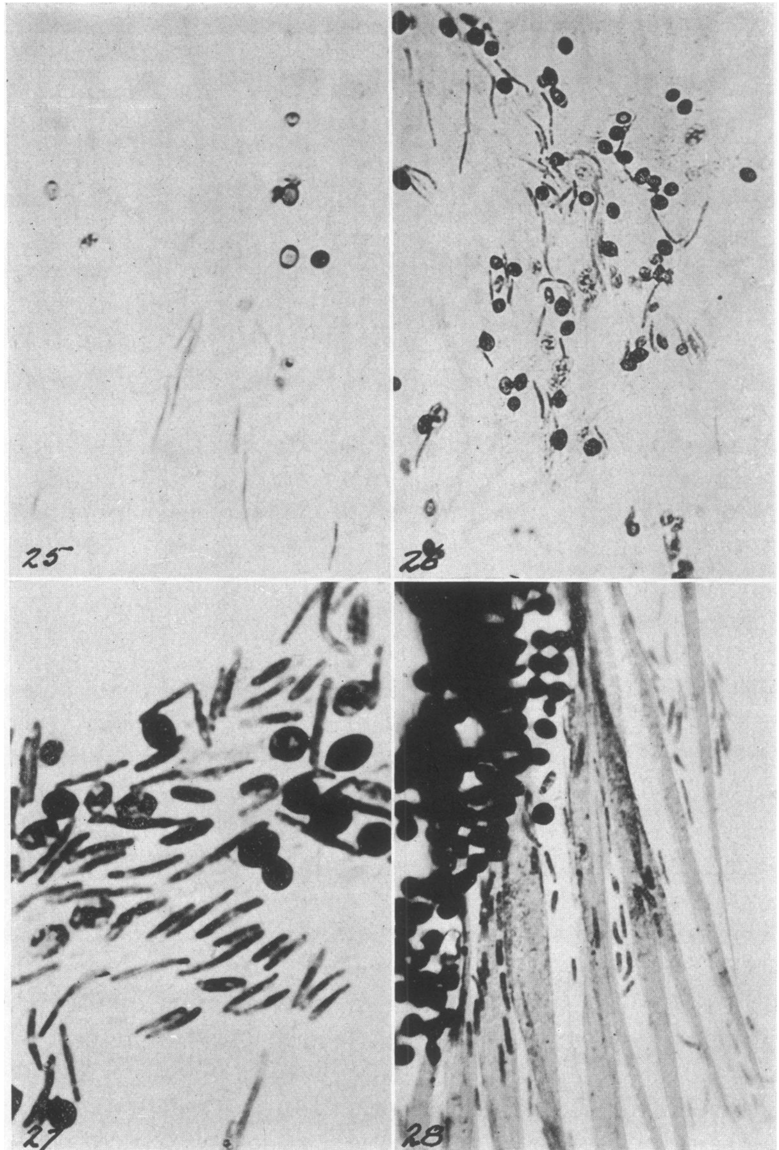

FIG. 25. Stages in spore formation. One cell (left) containing four deeply stained bodies. Iron-hematoxylin. 1160×.

FIG. 26. Various stages in sporulation. Iron-hematoxylin. 1160×.

FIG. 27. Same. Gentian violet-iodine. 1860×.

FIG. 28. Migration of cells toward fruiting center. The lightly stained material appearing in "folds" is the slime upon which the cells rest. (Wrinkled during preparation). Matured spores on left; cells in vegetative and transitional stages at center. Note binucleate cell above left of center. Iron-hematoxylin. 1160×.

nates. By the time the newly emerged cell is ready to separate from the old spore wall (fig. 2, S) the nucleus has divided, producing a rod-shaped binucleate cell. Unless a chromosomal union has been affected during the spore stage this nuclear split is a reduction-division for the purpose of producing vegetative cells with the typical number of chromosomes. Following the division of the nucleus the cell elongates, constricts at the middle, and divides, producing two typical vegetative cells, each with a single compact mass of nuclear material in the center.

If any sexual union were to be noted it would of necessity occur immediately following the post-germinal nuclear division, resulting in four haploid cells. The union of a pair of these cells would be necessary for the formation of typical vegetative cells. However, no indication of cell conjugation has been observed, and it is thought that this step, which is typical of some of the fungi, is not included in the life cycle of *M. xanthus*.

SUMMARY

A new species of *Myxococcus* producing an orange-colored fruiting body is diagnosed and described, and the name *Myxococcus xanthus* proposed.

Methods of growing the bacterium on media, utilizing rabbit dung as the source of nutrient material, are described.

Microscopic slide preparations were made by pressing clean, sterile cover slips down on the growing colony. The adhering cells are fixed, stained and mounted in neutral balsam.

The life cycle of *M. xanthus* is relatively complex. In the vegetative stage the cells are long, flexible, single rods, that move over the surface of the substrate by a crawling or creeping motion. They are grouped on the substrate in small clumps, their long axes parallel, and move in clockwise paths as a unit toward the margin of the colony. As the cells go into the spore stage they shorten and become perfectly spherical by the time they are imbedded in the slime of the fruiting body. Germination is by a process analagous to budding.

Evidence is presented supporting the theory of a compact or condensed nucleus. In the vegetative phase the nucleus is a

single compact mass of nuclear protoplasm that divides prior to cell fission. It has a marked affinity for nuclear dyes, and is Feulgen-positive. During the transitional phase the nucleus breaks up into four chromosomes that are stained by gentian violet and iron-hematoxylin. In the prophase the chromosomes are shown to be made up of chromomeres. An autogamous fusion of chromatin material occurs before the mature spore has been formed, and nuclear division, presumably meiotic, takes place during germination of the spores.

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