CYTOPHAGA COLUMNARIS (DAVIS) IN PURE CULTURE: A MYXOBACTERIUM PATHOGENIC TO FISH[1]

LAURA GARNJOBST

Aquatic Biologist, U. S. Fish and Wildlife Service, Kearneysville, West Virginia

Received for publication August 24, 1944

A rod-shaped bacterium capable of unusual motility was described by Davis in 1922 as the cause of a distinct dermal disease of numerous fresh-water fishes. He named the organism *Bacillus columnaris*, but owing to changes in the system of classification since that time (Bergey *et al.*, 1939) it was automatically excluded from this genus. There is at present also a better understanding of other forms having this peculiar creeping mode of locomotion without flagella. The vegetative rod form, thin cell membrane, flexibility, and the aflagellar progressive movement on surfaces now indicate the complex (higher) myxobacteria or closely related species.

It is the purpose of the present paper to give a more extended account of this interesting and important parasite—an account made possible by its isolation and cultivation in a variety of media. Special emphasis has been given to the study of its life cycle, but no fruiting bodies or microcysts appear to be formed. Therefore, the bacterium has been assigned to the family *Cytophagaceae* (Stanier, 1940), genus *Cytophaga*. If this position is accepted, *columnaris* becomes the first member of the *Cytophaga* group (Stanier, 1942a) known to be an animal parasite. Its nutritional requirements consequently should be of general interest.

TECHNIQUE

The initial colonies of *C. columnaris* were obtained from infected bullheads (*Ameiurus nebulosus*) taken from a pond at the Leetown laboratory of the U. S. Fish and Wildlife Service in July and August, 1943. To eliminate most of the other, usually more rapidly growing, bacteria from the original inoculum, the following method was found best. A flake of debris was removed from a selected area of the lesion, deposited at one side of a large drop of sterile spring water on a slide, and left until the rods had swarmed away from the mass for some distance. The debris was then removed with a pipette, and some of the remaining bacteria were transferred with a loop in the usual way without further dilution.

Colonies appeared both in nutrient gelatin and in nutrient agar plates in four or more days at temperatures between 13 and 25 C. In all, 15 colonies were removed from semisolid nutrient gelatin into the following liquid media: (1) fish extract with 1 per cent peptone added; (2) Difco proteose peptone (1.0%, 0.25%); (3) tryptone (0.5%); and (4) proteose peptone containing hemoglobin (0.5%). Growth occurred in all except medium (4) but very little, if any, in (1). Four of the original cultures from these colonies probably were pure from the

[1] Published by permission of the Director, U.S. Fish and Wildlife Service.

beginning, since serial transfers were made at irregular intervals for six months without contaminants making their appearance. Strains from five colonies were further purified and maintained so, but no significant difference has been noted between them, or between them and the strains not further purified, in behavior or appearance of the bacteria during the period of study (August, 1943, to February, 1944).

The pathogenicity of the bacteria grown in pure culture was tested by inoculation into slight surface injuries in healthy sunfish. Within certain temperature ranges, the characteristic lesions of this and other kinds of fishes in natural and induced "columnaris infections" were readily produced, followed by death of the fish in 100 per cent of the cases (Davis, report in preparation). The bacteria in culture also retained their distinctive behavior, including the columnar swarming movements in water mounts. For these reasons there is no doubt that the organism isolated is the same as the long flexible rod found in abundance and in almost pure culture in the lesions of fish.

Stock cultures were maintained in a 0.5 per cent proteose peptone or tryptone solution (initial pH, 6.3 to 7.1). The bacteria were sometimes difficult to remove from an agar surface after about 24 hours of growth and, therefore, a liquid medium was preferred, especially in the early stages of the study. Each strain was maintained at two different ranges of temperature, 13 to 18 C and 20 to 25 C. Some of the tubes at the lower temperatures remained viable for more than 30 days.

Preliminary experiments were made in an effort to obtain an entirely adequate stock medium. Eight different media, including solutions of peptones, yeast, and *Elodea* extracts were prepared at optimum concentrations previously ascertained and the amount of growth obtained compared. Of these, proteose peptone and tryptone were selected as suitable basal media (pH about 6.8) for testing other substances which might be added. Small amounts of glucose or lactose added did not appear to give more rapid or extensive growth. Yeast extract added to tryptone solutions (known to be low in vitamins) delayed the appearance of turbidity as compared with the controls. A solution of vitamins (thiamin, nicotinic acid, calcium pantothenate, riboflavin, *para*-aminobenzoic acid, inositol, and pyridoxine) with or without glucose did not produce any noticeable effect, but there was no period of lag at the beginning.

The bacteria were fixed and stained in a variety of ways from several kinds of media. In addition to the usual routine bacteriological methods, cytological procedures without drying at any stage were adapted to the organism. Zenker's or Worcester's fixing fluids gave good results, followed by staining in hematoxylin (Heidenhain's or Ehrlich's) or basic fuchsin. Other fixatives tried out were Bouin's, Schaudinn's, and Da Fano's cobalt nitrate. Differential staining methods used, in addition to Gram's, Neisser's acid methylene blue and Bismarck brown, and Epstein's granule stains, were Mallory's tricolor after Zenker's fixing fluid, Giemsa's dry, and Dobell's (1911) wet method using Bouin's fixative, Winogradsky's (1929) phenol-erythrosine and gentian violet after fixation in osmic acid fumes, and finally Robinow's (1942) modification of Feulgen's "nu-

cleal" reaction. These methods proved useful from a comparative viewpoint, especially taking into account the structure as observed in the living bacteria.

Other techniques and staining methods are mentioned in connection with the structure or experiment concerned. Unless otherwise stated, all measurements given were made on living material.

MORPHOLOGY

The most common form of *C. columnaris* observed in nature is the long, slender, flexible rod, with rounded ends, 5 to 12 microns long and about 0.2 microns wide (fixed and stained cells), first described by Davis (1922). Three other forms were discovered in pure culture: (1) long filaments, segmented or unsegmented, (2) branched cells, and (3) "ring forms," simple and complex. This study has also extended the dimensions. Filaments were found which measured as high as 163 microns in length, and the smallest cells were only two microns long. The vegetative cells (figure 1, nos. 1 to 8; figure 2, nos. 46, 48) in peptone media averaged about 8.0 microns; width measurements varied from 0.2 to 0.4 microns. On nutrient agar these cells averaged about 10 microns in length. None of the rods tended to appear spindle-shaped at any time.

Multiplication is by transverse division, usually into two cells of approximately equal length. The division appears to be by cell "constriction" (Stanier, 1940, 1942a); in wider cells, such as *Cytophaga rubra*, this detail undoubtedly is more readily determined. In living material, recently separated cells sometimes seem attached to one another by a connecting thread, but it is difficult to be certain that the strand is not secreted slime. Before separation of the cells, the rod often becomes bent at the mid-point. Such V-shaped structures were everywhere in evidence in young fluid cultures, but they also appeared when cells near the period of elongation were brought into water from an agar plate culture. The change seemed to stimulate cell division, and the whole process till complete cell separation could then be observed under the microscope repeatedly. It is not meant that cells necessarily always bend in this manner prior to separation, but it is very characteristic of this species. It is not accidentally brought about, for instance, in transfer, but rather by movement of the cells themselves (see section on movement).

The filaments are unsegmented or segmented, depending upon the time when they were removed from a culture. In older cultures cell division as well as cell separation appears to be suppressed. Upon transfer to a fresh medium the unsegmented filaments often become segmented and separate into cells, usually of unequal length at least at first, the final result being cells of small size (2.0 to 5.0 microns). Under circumstances of more or less precipitous division of filaments, branched cells were first noted (figure 1, nos. 9 to 14; figure 2, no. 49). The dense layer of filaments lying parallel to one another, as they generally are, would of course be more durable with branched than with unbranched filaments alone. If they are involution forms they nevertheless have been seen undergoing flexion and in creeping movement. Two or more branched forms in one field of the microscope were not uncommon in some unstained as well as stained preparations.

Krzemieniewska (1930) described similar branched cells in *Spirochaeta cytophaga*[2] and found them to have nothing in common with the branched cells of the actinomycetes.

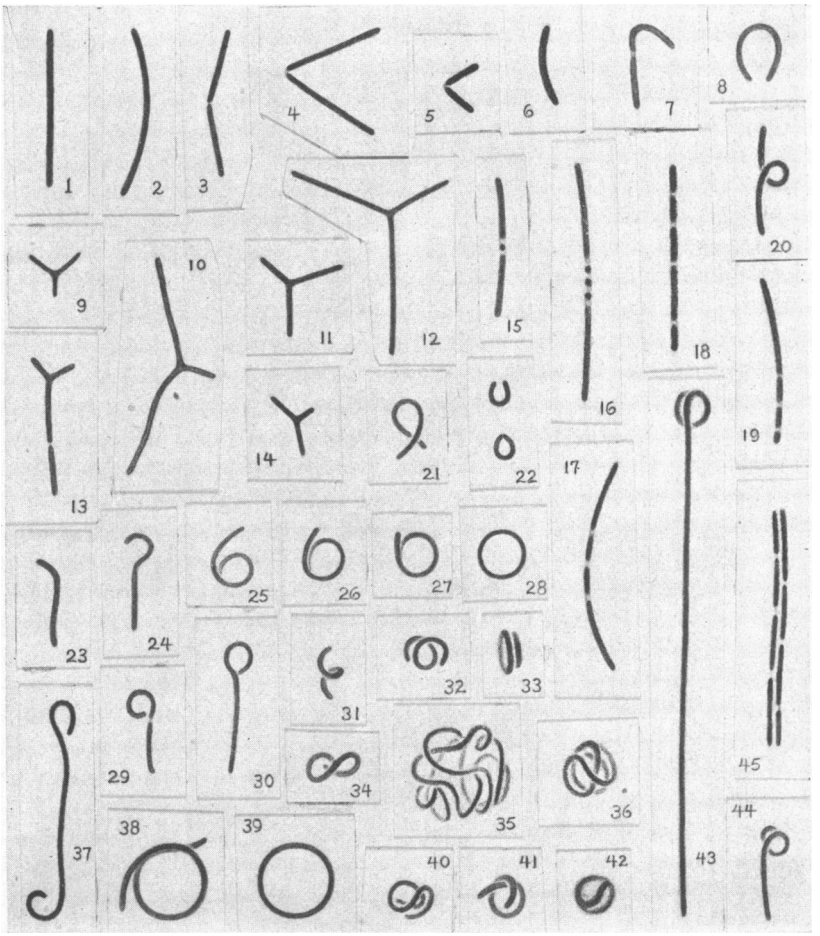

FIG. 1

All drawings were made with the aid of a camera lucida

Nos. 1–45 incl. *Cytophaga columnaris*. Drawings made from fixed and stained preparations except as noted below. Bacteria grown on peptone nutrient agar. × 2600, reduced $\frac{1}{5}$.

Nos. 1–8. Vegetative cells, from living material. Nos. 3–5, stages of division.

Nos. 9–14. Branched cells. Nos. 11–12, from living material.

Nos. 15–19. Pseudobarred or beaded cells (probably artifacts).

Nos. 20–44. Coil or ring involution forms. Nos. 20–27, from living material. No. 35. Complex ring form, composed of one long filament. Nos. 35–43, cells stained with vital neutral red.

No. 45. Section of two segmented filaments.

C. columnaris cells do not retain stains as readily as many other bacteria, although after fixation in Zenker's fluid and staining for several hours in basic

[2] *Sporocytophaga myxococcoides* (Hutchinson and Clayton emend. Krzemieniewska), Stanier (1940).

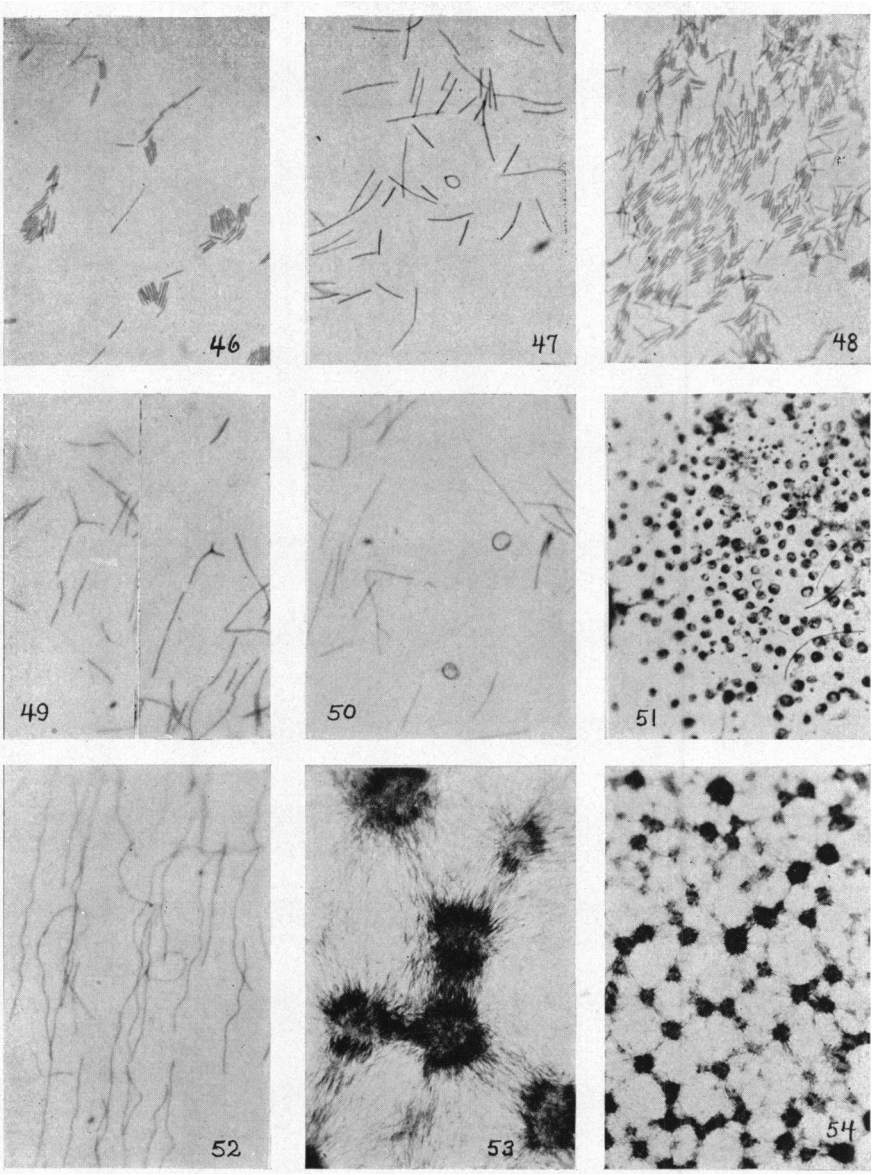

FIG. 2. PHOTOMICROGRAPHS OF FIXED AND STAINED *Cytophaga columnaris* PREPARATIONS

Nos. 46 and 48. Vegetative cells from surface of agar plate culture. Zenker's fluid; basic fuchsin. × 680.

No. 47. Vegetative cells from peptone agar showing one simple ring form. Worcester's fluid; basic fuchsin. × 680.

No. 49. Two branched cells from 0.5 per cent tryptone. The larger cell is in an early stage of division. Worcester's fluid; basic fuchsin. × 1075.

No. 50. Coiled rods. Zenker's fluid; Ehrlich's hematoxylin. × 1075.

No. 51. Late stages of coiled cells (ring or coccus involution forms), with a few rods present. Worcester's fluid; Heidenhain's iron-alum hematoxylin stain. × 680.

No. 52. Long, undulated involution forms from an old culture. Worcester's fluid; Heidenhain's iron-alum hematoxylin stain. × 1075.

Nos. 53 and 54. Pellicle removed from an agar surface culture, showing pattern formed by the bacteria. Note the arrangement of bacteria about the clusters. In no. 51 in the center of the larger clusters can be seen a thicker patch of slime to which the rods adhere; the photograph was taken at a level near the base of the clusters. No. 51, × 340; no.52, × 170.

fuchsin, more deeply stained rods were obtained. The differential staining methods, such as Robinow's modification of Feulgen's technique, Winogradsky's stain, and Heidenhain's iron-alum hematoxylin, revealed no internal structures.

Invariably the living as well as the stained cells are homogeneous in appearance. This was found to be true also by Davis (1922). No colored particles appeared after application of scarlet R or Sudan III in 80 per cent alcohol. Also, no color reaction could be obtained in the living cells with the vital stains methylene blue or neutral red. On the other hand, the dilute solutions of these dyes stained the secreted substance vividly, producing a beautiful effect—colorless, living, moving cells adhering, often only at one end, to a bluish-purple or red substance, depending upon which stain was used. A test for volutin described by Meyer (1912), in which cells are first stained in methylene blue and then treated with 1.0 per cent sulphuric acid, left no blue color within the cells.

Some cells fixed by drying have a barred or segmented appearance, but this is not comparable to the transverse bands described by Krzemieniewska (1930) since in *C. columnaris* there is no difference in stainability. The protoplasm within the cell membrane is changed so that it appears to be segmented or beaded as shown in nos. 15 to 19 (figure 1). It is often noted that one to three beads (that is, granules extending the width of the cell) are present at the mid-point of a rod likely to be near division. Cells from the same source stained (upon death) with vital methylene blue or neutral red or fixed in the sublimate mixture, Zenker's or Da Fano's fluids, show less distinct but similar structural details in some cells. However, preparations made by pressing a coverslip on the cells growing on an agar surface and fixing the readily adhering cells in Zenker's fluid promptly without drying preserved most nearly the conditions of the living cell (figure 2, nos. 46, 48), and in these the structural effects just described are usually absent, although normal (that is, at least viable), segmented filaments occur. It is suggested that the pseudobarred or beaded conditions probably are produced at the time of fixation, and more often if the cells are near division (single or multiple). The protoplasm at this period (immediately before and after division) might be more fluid generally throughout the cell or at certain points. None of the differential stains tried produced differences in color or in intensity of stain between any such granules or segments present. The possible formation of conidia was considered.

Under certain not specifically known circumstances rod-shaped cells become irreversibly coiled (figure 1, nos. 20 to 44; figure 2, no. 50). Although it has long been known that a myxobacterial cell can bend, for instance in the form of a ring, Stapp and Bortels (1934) were the first to suggest that the peculiar "Krümmungsbewegungen" might be pathological in character. Stanier (1942b), on the other hand, stated emphatically that the flexing movements are not symptoms of degeneration, as believed by Stapp and Bortels, but are "always of very marked occurrence in the majority of young, healthy cells which are lying in contact with a surface" (p. 155). In *C. columnaris* normal flexing movements occur and were, indeed, very often observed (see section on movement), but these do not preclude irreversible coiling of cells. The usual fate of cells which remained coiled

appeared to be a series of involution or degenerate stages, from distinct, readily recognizable ring forms (simple or complex) to rounded bodies of various sizes, depending on the size of the cells which coiled, and finally to amorphous granules. After coiling, a veil of secreted slime often became associated with the cell so that, especially in the simple rings and in later stages of more complex rings, the whole presented the appearance of a coccus-shaped cell (figure 2, no. 51). One cannot escape the impression that it would be easy to confuse these stages with microcysts. They stain deeply in hematoxylin, and after the readily recognizable coiled stage the deeply staining part is irregularly distributed. Finally, in the irregular granules, stain is no longer retained to any great extent. The staining property of these involution forms call to mind the so-called pycnotic nuclei and their later stages of degeneration.

Coiling could be readily induced by mounting bacteria from liquid media or an agar surface in water. With or without a coverslip and usually within about ten minutes, coiling took place. In one such instance under a coverslip as many as 20 to 40 rings could be counted in any microscopic field. This number, however, was uncommon in preparations without a coverslip added. The degenerative stages sometimes were reduced so that within about one hour nothing remained but small homogeneous-appearing droplets, and these stained deeply in hematoxylin.

Coiling is not a function of a definite stage; but it appears rather to be due to certain environmental conditions. Cells of various sizes and cells undergoing division were observed to coil; even very long cells produced ring forms, and these were large and often complex (figure 1, nos. 35, 36).

Whatever their meaning the ring involution forms are important in a study of this kind: they are conspicuous and, as stated, the coccuslike stages could easily be confused with microcysts. Their mode of formation, however, is entirely different from that described for microcysts (Baur, 1905; Krzemieniewska, 1930; Stanier, 1942a; and others), for there is no shortening of rods to form the coccus stage. The presence of a few simple rings is very common, but in only one tube, containing 0.5 per cent of tryptone, did practically all the cells assume various simple and complex ring forms. When the coccuslike forms were found in great abundance, there were invariably present also at least a few rods. One exception observed was a plain gelatin culture. Experiments were made to test viability and these are described below.

MOVEMENT

Studies have been made of the movements of myxobacteria, notably by Baur (1905) and Stanier (1942b); certain differences are stressed in the following brief account of movements in *C. columnaris*. The bacteria were observed in water mounts and on nutrient agar surfaces. In a liquid nutrient medium on a glass slide, movements were rarely seen unless the nutrients were very dilute.

The rate of creeping locomotion in water on a glass surface (with the temperature uncontrolled but approximately between 20 and 22 C) was found to be relatively high (12 to 94 μ per min; average of 14 readings, 60 μ per min) as com-

pared with that on nutrient agar, and as reported for the marine species (30 μ to 50 μ per min). In rate of movement, *C. columnaris* resembles the soil cytophagas in which the rate has been estimated to be approximately 150 μ per minute, without temperature control (Stanier, 1942a). The rate, however, varied greatly from barely perceptible to rapid.

In following the course of a cell one might see it glide over other cells, stop at times, and then resume movement, usually in the same direction, although the course was not necessarily in a straight line. The direction of movement was occasionally reversed (1) by creeping with the opposite end foremost, for instance upon contact with an obstruction, or (2) by a swinging movement (that is, the rod taking suddenly a perpendicular and then a horizontal position), the same end being foremost in the resumed gliding but pointing in the opposite direction. On a moist agar surface such freedom of movement was probably prevented, but flexion was possible so that the direction was occasionally reversed (3) by bending.

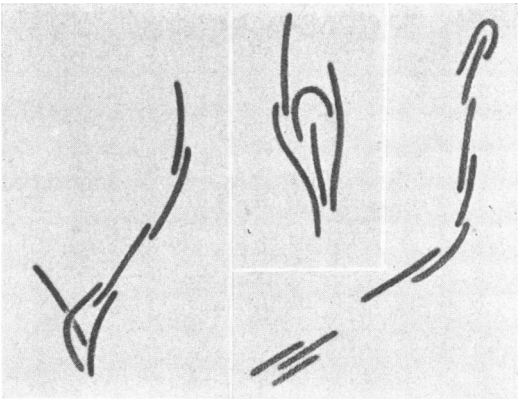

FIG. 3. COLUMNAR MOVEMENTS ON NUTRIENT AGAR SURFACE
Zenker's fixative; basic fuchsin. × 2600 reduced $\frac{1}{3}$.

Permanent records (figure 3) of movements of this kind were obtained when a coverslip was pressed on the bacteria swarming on an agar surface and the whole straightway immersed in Zenker's fixing fluid.

Movements in water were of special interest. In addition to those already described, peculiar rotary or waving movements, sometimes combined with flexion, were observed in rods which had suddenly assumed a perpendicular position on glass. The waving was so regular that automatically one began to count. The result of counts made on several different individuals are: 111, 218, 193. The horizontal position was suddenly resumed and sometimes just as suddenly the rod became perpendicular again to repeat the process. Usually the rod remained fairly rigid during the movement. On the other hand, one cell was less rigid, and in this the beat was irregular and rapid so that accuracy in counting the revolutions was impossible. The circle described by the free end was not complete and the rate dwindled noticeably just before the cell dropped to the horizontal position. These movements, which seem fantastic in so simple an

organism, are nevertheless fairly similar to those often seen when the bacteria emerge from a mass taken from a fish lesion or from an agar surface, but in these isolated individuals it was much easier to study them. Of all the movements observed this type is the only one which would seem to require flagella, but flagella were not discovered by special staining methods, such as Loeffler's and Fisher and Conn's, or relief stains using nigrosine or China blue. Since the electron microscope has revealed flagella in a spirochete, *Spirocheta pallida* (see Marton, 1943), the question should perhaps be left open.

It was interesting to observe cells in active movement undergoing division. When the rod became bent to a V-form, the movement became confused, and this undoubtedly aided in further bending and separation of the two cells. In one instance it was always one cell which moved; the other was bent above the moving cell and so carried in the same direction. The movement was even more confused when a longer rod became bent in two places. It is not impossible, though to the present investigator rather unlikely, that V-formation can occur in the absence of division. Benecke (1912) stated as follows: ". . . die normalerweise gerade, stäbchenförmige Myxobakterienzelle kann sich kreisförmig biegen oder auch zusammenknicken . . ." (Stanier, 1940, p. 620), an indication that the phenomenon has been observed in other myxobacteria. Baur (1905) in fact described it but did not associate it with fission.

The longer the cells become the less likely are they to be seen in motion. Even so, cells 20 microns long without any signs of division were observed to creep rapidly and characteristically on glass in a water mount. Long cells in a filamentous mass were seen undergoing a restless, rotary type of motion in entirely undisturbed areas of an agar plate.

CULTURAL CHARACTERISTICS

Liquid media. In the vegetative stages the rods appear rigid (turgid?). When not in movement they are straight or slightly curved (figure 1, nos. 1, 2) and readily cling to any small particle present, or to one another. The resulting clusters or stars, and individual cells as well, are distributed throughout the medium. In some instances clusters were found attached to the side of the tube where they formed distinctive colonies (figure 4), superficially resembling minute sea urchins. They ranged in size from microscopic dots to visible objects needing no magnification. In older stages the cells swarmed from the enlarged, rounded, or irregular mass, with the result that often papillaelike clusters projected from the entire exposed surface. These projections varied in number and eventually elongated into columns of organisms; or, rounded tips were formed which became detached into the medium as spheres.

A surface film was formed in some tubes, and there was considerable piling up of cells at the bottom, particularly at the lower temperatures (13 to 18 C). In such tubes, when shaken, a large spiral formed from the bottom layers, as would be expected, since the strands are composed of cells (some of them long) adhering to the glutinous secretion. A distinct yellow color often developed on the surface film or in flakes of declining cultures. A yellow ring was formed in fish extract.

Some of the bacteria, both long and short, were coiled into rings, especially at the higher temperatures, and masses of these were found in various stages of degeneration. Other remaining cells seemed finally to have lost their turgidity (involution forms), for they were twisted in any manner and undulated (figure 2, no. 52), and the threads were noticeably thicker.

Solid media. *C. columnaris* grows fairly well on an agar surface, such as Difco nutrient agar. The surface pattern formed by the bacteria varied, of course, depending in part upon the consistency of the agar and the amount of moisture present in the air above, or below, the surface. If the agar was firm and little moisture present in the air, no growth occurred at all. Just the right

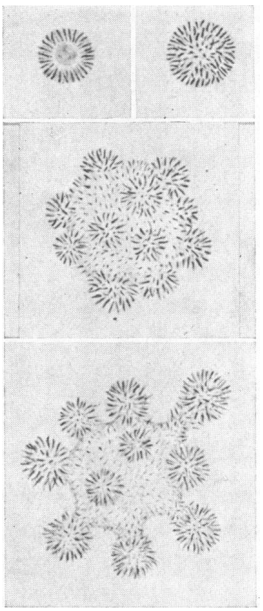

FIG. 4. CLUSTERS (OR STARS) FORMED IN 0.5 PER CENT TRYPTONE SOLUTION
Living material. Semidiagrammatic. × 125.

amount was required. Until this was realized, the growth response of the organism on agar (and gelatin, to some extent) seemed erratic.

Within the first 24 hours of growth following surface streaking from a liquid culture, a pellicle was readily removed (peeled) from the agar. This pellicle[3] was uneven in thickness and the pattern formed by the bacteria adhering to it was related to the movement of the organism on a solid nutritive substrate. After 24 hours, more or less, the pellicle usually became closely adherent to the agar and could not be successfully removed. The bacteria as they migrate from a

[3] The pellicle, composed of bacteria and the yellowish secretion, gives a blue color reaction with concentrated sulphuric acid. Small flakes almost immediately are haloed by a bright blue color which increases in extent. In a few minutes the blue color begins to fade into a dirty yellow and then becomes distinctly reddish in color.

given point both tend to spread over the surface in columns and to form aggregates or clusters (figure 2, nos. 53, 54) from which other migrations occur if there is considerable moisture present; otherwise, the aggregates remain small and branching, interlacing columns are formed. Variations between these two extremes of pattern occurred also. The layers of bacteria increase because the organisms continue for some time to migrate back and forth and to multiply along the "paths" taken. If the inoculation of the agar surface has been such that colonies were well separated, the original points of growth were recognizable for many hours after the whole surface of the plate had been overgrown with bacteria.

Samples removed from a plate about 18 hours after inoculation usually showed elongated cells in some portion of the growth. These gradually increased in number until there was formed a more or less continuous, felted cover with many of the filaments lying in parallel rows or swirls. When samples were removed during the early period of elongation and mounted in water, the cells invariably began swarming from the mass in precisely the same manner as that described and figured by Davis in samples taken from lesions of fish. Samples were then taken at hourly intervals for 10 hours, and it was discovered that finally this behavior no longer took place. The cells had become long. When transferred to nutrient media they reverted to small cells, but not in all cases. Just when the filaments become nonviable (that is, when no further reproduction occurs) is not readily determined, since it is difficult to test many individual filaments. If all rods which divide into more than two cells are involution forms, they may nevertheless be of some importance to the species. It is possible that the suddenly increased numbers at high temperatures in ponds or streams may be enhanced by the multiple division if at the same time nutrients are available.

In declining agar plate culture, the color *en masse* becomes changed from pale yellow to a deeper, old-gold shade, sometimes with a tinge of orange. Samples taken showed rings and ring involution forms in patches. A characteristic sickening odor is given off by growth of the bacteria on Difco nutrient agar, and this was most noticeable after 24 hours or more. No reference to an odor of this kind was noted in the literature regarding the myxobacteria.

To test the presence of resistant stages, inoculations into liquid media were made daily from an agar plate culture. After 48 hours, the inoculum contained elongated cells and rounded bodies. With time, the former decreased in number whereas the latter increased in number. The time to the appearance of turbidity in tubes of tryptone solution increased, and after the eighth day the solution in the inoculated tubes remained clear. This experiment was repeated with rounded bodies formed in nutrient gelatin with the same result in seven days. In one tube rounded bodies only were present in the gelatin. No growth was obtained in the tubes inoculated. Both unstained (living) and stained preparations made from this and similar material showed that the structure of the rounded bodies resembled in every way later stages of ring involution forms.

Colonies. Young surface agar colonies (figure 5) are flat, colorless or pale yellow, and intricately branching. In the spreading outer zone, particularly, the

branches often appear discontinuous owing to the swarming of cells in groups. The pattern in the peripheral zone for some distance is continually modified. Branches are sometimes withdrawn, and an entirely new pattern appears; or a group of rods becomes stranded at a distance, sometimes far enough to begin a new colony. The older, central portion of an old colony formed from a minute

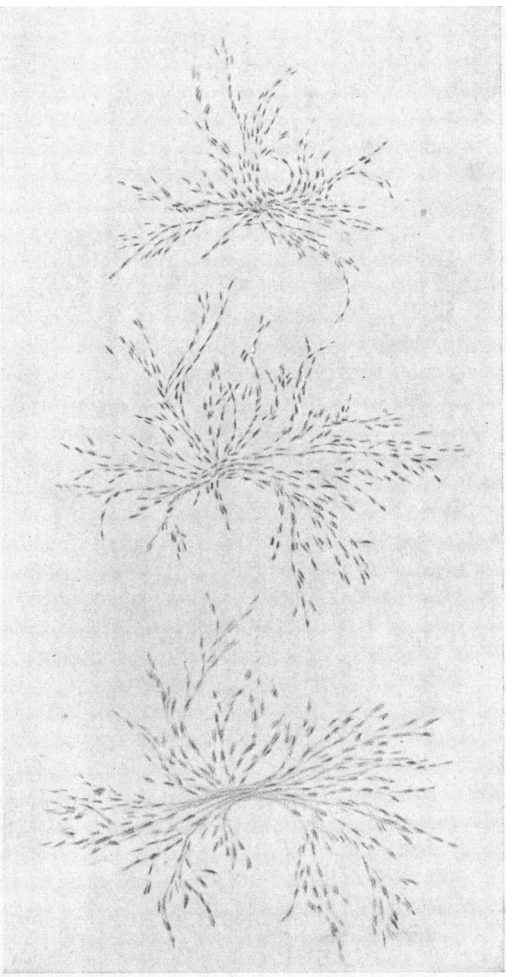

FIG. 5. SURFACE PEPTONE AGAR COLONIES (72 hr.)
Semidiagrammatic. × 250, reduced $\frac{1}{3}$.

inoculation at the center of an agar plate becomes warty in appearance, and the pattern of the surrounding zone gradually becomes fixed into layers of long filaments along the main branches. These colonies rarely exceed 5.0 cm in diameter. Colonies beneath the agar surface are massive, and globular or irregular. Sometimes projections are present, their formation depending upon the softness of the agar and the consequent migration of the cells if enough moisture is present. All

gradations of agar colonies were observed from entirely globular colonies to a condition in which a central globular mass was surrounded by the characteristic flat network of branches. In older stages the network was sometimes withdrawn, leaving a deep yellow, entirely spherical, surface colony.

Gelatin colonies resembled those formed on agar, but the bacteria soon liquefied the gelatin and therefore the colonies became irregular, filamentous masses. These colonies were readily removed *in toto* because the cells adhered to the slime secreted.

NUTRITIONAL REQUIREMENTS

Nitrogen. *C. columnaris* was found to grow satisfactorily in a medium containing only peptone. This is not surprising when one considers its luxuriant growth on the dermal cells of fish at optimum temperatures, 25 to 30 C. Experiments (at 25 C $\pm$ 0.5) to test whether or not the nitrogen requirements could also be met by supplying an inorganic source were negative. KNO_3 or $(NH_4)_2SO_4$ were supplied in concentrations ranging from 0.1 to 1.0 per cent in combination with 0.1 per cent glucose or lactose in Leetown spring water or the mineral medium used by Stanier (1942). The only growth which occurred was in the control tubes which contained also 0.1 per cent peptone, and this was of the order found in a 0.1 per cent solution of peptone alone.

Peptones are not required as such, however, since the organism will grow well, even after several transfers, in a mineral base to which hydrolyzed casein has been added. These experiments also showed that the organism probably synthesizes any necessary vitamins, because the hydrolyzed casein used was found to be free of vitamins. (For this preparation the author is indebted to Dr. E. L. Tatum, Stanford University, California.) Further experiments are now being made to discover which amino acids are required for growth.

Carbohydrates. Preliminary experiments using the ordinary standard methods had shown no clear-cut positive results for the utilization of sugar, although in tryptone solutions the organisms could tolerate a concentration up to 0.7 per cent of autoclaved glucose without atypical growth. As previously described, a dilute solution of neutral red produced a definite color change to red in the slime secreted by the bacteria. Any consideration of acid production in small amounts would have to take into account this apparently basic substance produced. Using glass filter-sterilized glucose in amounts ranging from 0.01 to 0.1 per cent in 0.1 per cent peptone, no detectable utilization could be demonstrated by qualitative methods.

Agar appeared not to be decomposed in any recognizable degree. The I-KI solution test recommended by Stanier (1941) also was negative. The addition of calcium carbonate to an agar nutrient medium did not give more extensive growth than a similar agar medium without this substance added. The cellulose decomposition tests were made with filter paper strips in (1) the mineral medium of Stanier, with an initial pH of 7.1, and (2) in media containing 0.1 to 0.5 per cent peptone, or 0.1 per cent peptone and the nitrate salts (0.1%). In (2) there was no evidence of decomposition microscopically within 17 days. Although no

growth of *C. columnaris* was obtained in (1) within 14 days, *Sporocytophaga myxococcoides* (from Dr. C. B. van Niel) inoculated into the same medium produced evidence of growth in six days and macroscopic decomposition of the cellulose on the tenth day after inoculation.

LIFE CYCLE AND SYSTEMATIC POSITION

In the classification of the *Myxobacteriae*, as revised by Stanier (1942), great emphasis has been placed on the production or absence of microcysts in the life cycle. There is a tendency in the literature, moreover, to regard descriptions without this stage as incomplete, which is not surprising in view of work in other fields and the long known life cycle of the higher myxobacteria. The spores in yeasts, for example, are not readily obtained in culture, and a medium used to produce spores in one species does not necessarily produce them in others (Henrici, 1930), yet this stage has been selected as a means of classification. Studies on the higher myxobacteria have shown that fruiting bodies and microcysts are often discovered under natural conditions and sometimes fail to form, or are less normal, in the same species in certain laboratory media (Beebe, 1941; Snieszko, McAllister, and Hitchner, 1943; and others). Although no such stages have been found in *C. columnaris* under natural conditions or in the media already described, in the present study it was considered important to make further tests using complex media.

Fish tissue autoclaved in small amounts for a minimum of time (5 to 10 minutes at 121 C), with or without agar, failed to produce microcysts in *C. columnaris* at several temperatures, including 30 C. Several kinds of eubacteria isolated from pond water (*Pseudomonas* and *Sarcina* species) were autoclaved and added to the medium or used alone in agar or water suspensions, according to the methods described by Beebe (1941), but these also failed to give positive results.

Snieszko *et al.* (1943) suggested that the pronounced proteolytic properties of myxobacteria were probably responsible for the observations made by other investigators and themselves that when the vegetative growth is more profuse, there is a greater probability that fruiting bodies will not develop at all, or will be autolyzed before development is complete. *C. columnaris* forms proteolytic enzymes, known from its rapid liquefaction of gelatin, clearing action in milk, and slow dissolution of autoclaved bacteria. In regard to the latter it may be mentioned that the organism will grow in a medium composed of insoluble material prepared from *Photobacterium* sp. (for preparation see Tatum *et al.*, 1942) in a mineral solution. Scantier growth which permitted development in spite of enzyme production in two species of *Myxococcus*, according to Snieszko *et al.*, did not bring forth fruiting bodies or microcysts in *C. columnaris*. In tryptone or proteose peptone media with 0.1 per cent agar *C. columnaris* produced a heavy pellicle, but no fruiting bodies were formed. Ring involution forms were abundant.

There are now at least six (probably nine) myxobacteria in which microcysts are definitely considered absent (Stanier, 1942; Fuller and Norman, 1942). The present species differs from these cytophagas in several respects, the most important being that cellulose is not decomposed to any recognizable degree. This

statement leaves out of account two soil cytophagas, very briefly described by Fuller and Norman (1943), which also do not attack cellulose; but the fact that *C. columnaris* is an animal parasite probably would distinguish it from these soil forms. These differences, indeed, might warrant the creation of a new genus. The hesitancy to do so seems justified for the present because of the small number of species in the *Cytophaga* group and the present status of this field of research. Stanier's temporary groups might well be extended to three, including a fresh-water group. Whether or not *C. columnaris* is a true water form is of course not definitely known at the present time.

DIAGNOSIS

Cytophaga columnaris (Davis) Garnjobst; *pro synon. Bacillus columnaris* Davis, 1922

Morphology: Highly flexible, singly occurring rods, 0.2 to 0.4 by 2.0 to 12.0 microns, capable of columnar, aflagellar, progressive movement on surfaces. Length very variable, average about 8.0 microns. In older cultures, cells from 12.0 to 20.0 microns are common. Branched cells are sometimes found. Gram-negative. Cells stain evenly by Giemsa's, Winogradsky's, and Feulgen's stains, and by Heidenhain's iron-alum hematoxylin. Thickened filaments and coiled (ring or coccoid) involution forms usually occur in old cultures. Star-shaped aggregates of cells form in liquid media and on agar with considerable moisture present.

Peptone agar plate: Growth begins as a pale yellow spreading swarm which rarely exceeds a diameter of 5.0 cm at any time. The branching, anastomosing columns of cells form a continually changing pattern, but gradually the pattern becomes fixed, beginning at the center, or older portion, and progressing outward. Warts and ring involution forms appear in the older portions. After 3 to 4 days, the color becomes distinctly yellow or old gold (sometimes with a tinge of orange) with a glistening surface.

Non nutrient gelatin: Rapid, stratiform liquefaction.

Liquid media: Growth is turbid and silky, sometimes with a pellicle which becomes yellow with age; at 13 to 18 C particularly, cells become piled up at the bottom of the tube.

Utilizable carbon sources: Proteinaceous materials are the only ones known. Cellulose, starch, and agar not attacked.

Utilizable nitrogen sources: Peptone and hydrolyzed casein are the only suitable ones known.

Hydrogen sulfide formed.

Catalase positive.

Indole not formed (Gnezda test).

Salt concentration range: To 0.3 per cent in 0.5 per cent tryptone solution.

Strictly aerobic.

Optimum temperatures: 25 to 30 C.

Source: Dermal lesions, fresh-water fishes.

Habitat: Cutaneous and connective tissues of fish.

SUMMARY

Bacillus columnaris Davis, 1922, a dermal parasite of fresh-water fishes, has been isolated and grown successfully in peptone media and in hydrolyzed casein added to a mineral base. Its morphological characteristics, aflagellar type of creeping motility, and absence of microcysts in the life cycle place this organism in the order *Myxobacteriales*, family *Cytophagaceae*. Reasons for including *B. columnaris* in the genus *Cytophaga* are presented. This species was found to differ from other members of the genus in not attacking cellulose to any extent, in its parasitic nature, and in other minor respects.

I wish to express my thanks to Dr. H. S. Davis for helpful advice during the course of this work, and also for making the photomicrographs.

REFERENCES

BAUR, E. 1905 Myxobakterien-Studien. Arch. Protistenk., **5,** 92–121.

BEEBE, J. M. 1941 Studies on the myxobacteria. 2. The rôle of myxobacteria as bacterial parasites. Iowa State Coll. J. Sci., **15,** 319–337.

BERGEY, D. H., BREED, R. S., MURRAY, E. G. D., HITCHENS, A. P., *et al.* 1939 Bergey's manual of determinative bacteriology. 5th ed. Williams & Wilkins, Baltimore.

DAVIS, H. S. 1922 A new bacterial disease of fresh-water fishes. U. S. Bur. Fisheries, Bull., **28,** 261–280.

DOBELL, C. C. 1911 Contributions to the cytology of the bacteria. Quart. J. Micro. Sci., **56,** 395–506.

FULLER, W. H., AND NORMAN, A. G. 1943 Observations on some soil cytophagas. J. Bact., **44,** 256.

HENRICI, ARTHUR T. 1930 Molds, yeasts and actinomycetes. J. Wiley and Sons, New York.

KRZEMIENIEWSKA, H. 1930 Le cycle évolutif de *Spirochaeta cytophaga* Hutchinson et Clayton. Soc. Bot. Poloniae Acta, **7,** 507–519.

MARTON, L. 1943 The electron microscope in biology. Ann. Rev. Biochem., **12,** 587–614.

MEYER, A. 1912 Die Zelle der Bakterien. G. Fischer, Jena.

ROBINOW, C. F. 1942 A study of the nuclear apparatus of bacteria. Proc. Royal Soc. (London), B., **130,** 299–324.

SNIESZKO, S. F., MCALLISTER, J., AND HITCHNER, E. R. 1943 On the biology of certain myxobacteria. Quart. Bull. Polish Inst., Arts Sci. America, Apr., 1–13.

STANIER, R. Y. 1940 Studies on the cytophagas. J. Bact., **40,** 619–634.

STANIER, R. Y. 1941 Studies on marine agar-digesting bacteria. J. Bact., **42,** 527–558.

STANIER, R. Y. 1942a The *Cytophaga* group. Bact. Revs., **6,** 143–196.

STANIER, R. Y. 1942b A note on elasticotaxis in myxobacteria. J. Bact., **44,** 405–412.

TATUM, E. L., GARNJOBST, L., AND TAYLOR, C. V. Vitamin requirements of *Colpoda duodenaria*. J. Cellular Comp. Physiol., **20,** 214.

WINOGRADSKY, S. 1929 Études sur la microbiologie du sol. Sur la dégradation de la cellulose dans le sol. Ann. inst. Pasteur, **43,** 549–633.