STUDIES OF FRESHWATER BACTERIA

II. STALKED BACTERIA, A NEW ORDER OF SCHIZOMYCETES[1]

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"*Pour se faire une idée suffisante sur les relations taxonomiques des bactéries, groupe devenues si important sous tant des rapports, il convient de ne pas se borner aux formes les plus habituelles et à cause de cela les mieux connues; il faut diriger les recherches sur des bacteries particulières, capables de donner quelques aperçus nouveaux sur la question de la morphologie du group en général.*"—ÉLIE METCHNIKOFF.

In the first paper of this series (Henrici, 1932) there were illustrated bacteria growing upon submerged slides which appeared to be attached to the slide by stalks. Since the publication of that paper a number of further studies have been made, in the course of which further types of stalked bacteria have been found. It has been determined beyond question that the fine filaments to which the bacteria are attached are actually stalks, because holdfasts have been found at their bases. A search of the literature has revealed descriptions of other types of stalked bacteria. While our study of these organisms is necessarily very incomplete, we feel that we have sufficient data on hand to warrant a preliminary discussion of these peculiar bacteria and their taxonomy.

To one unacquainted with these microörganisms, they might at first glance be considered as types of Myxobacteriales, which are also stalked bacteria in the sense that they form fruiting bodies upon stalks. The mechanism by which the stalks are formed in the two groups is, however, quite different. In the myxobacteria,

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the stalks are at first composed of bacterial cells, which arise by peculiar creeping movements of one cell upon another; the stalks are of macroscopic dimensions, and bear a very large number of cells at their tip. With the organisms here considered, each bacterial cell is borne upon an individual stalk, which is composed of gum or iron, and which is produced by a continuous secretion from one particular portion of the cell. They are exactly analogous to the stalked diatoms and protozoa. After cell division, the stalks may branch, so that in some cases a rather elaborate colony is eventually formed.

Such bacteria have been almost completely unknown to science because the standard methods of bacteriology will not reveal their presence. They cannot readily be fitted into any of the existing orders of bacteria. They are distinctive in their morphology, and a number of types have been found, sufficient to warrant the creation of a new order to include them. We propose the creation of such an order, to be called Caulobacteriales, from the Greek καυλός, a stalk.

STALKED BACTERIA PREVIOUSLY DESCRIBED

The following bacteria have previously been described as forming stalks: *Nevskia ramosa* Famintzin, *Bacterium pediculatum* Koch and Hosaeus, *Gallionella ferruginea* Ehrenberg, *Gallionella minor* Cholodny, and *Hyphomicrobium vulgare* Stutzer and Hartleb. A *Pasteuria ramosa* described by Metchnikoff, while not so distinctly stalked, shows strong morphologic similarities to certain of our stalked bacteria, and may well be included in the group. An unnamed organism described by Mabel Jones as possessing a single polar flagellum visible in slides stained by ordinary methods, was probably a stalked bacterium. The same, or a very similar organism, was reported by Omeliansky, and named *Bacillus flagellatus*. We believe that the *Vibriothrix tonsillaris* recently described by Tunnicliff and Jackson is also a stalked bacterium.

Nevskia ramosa

This organism was observed in 1891 by Famintzin in an aquarium in the botanical gardens at St. Petersburg. It formed a scum on the surface of the water, composed of globular, bush-like or plate-like colonies. The colonies were composed of gummy material arranged in dichotomously branched stalks arising from a common base, with the bacterial cells contained in the gum, a single cell at the tip of each stalk. The rod-shaped cells were set with their long axis at right angles to the axis of the broad lobe-like stalks.

The cells averaged 12μ in length, the length being 2 to 6 times the thickness. A distinct membrane could be demonstrated by staining with weak methyl violet, and in some instances concentric layers of capsular material were found surrounding the cells. Within the cells were highly refractile globular bodies varying in number and size. These were completely soluble in 70 per cent alcohol, and were thought to be composed of ethereal oils. The stalks dissolved rapidly in 1 per cent sodium hydroxide, setting free the cells which could then be readily stained and observed.

The cells multiplied by transverse binary fission, each daughter cell continuing to secrete its stalk, which thus produced the forked branching. At times the cells were seen to be set free from the stalks and to float away in the water. In this way the species is probably spread, such freed cells starting new colonies. All attempts at cultivation were unsuccessful.

Famintzin compared his organism to the palmellaceous alga, Urococcus, which similarly forms colonies of branched lobose stalks with the cells enclosed in gum at the tips, while Migula notes a resemblance to *Chaetophora endiviaefolia*. One might at first glance believe that Famintzin was dealing with an alga of some sort. The size recorded is rather large for bacteria, but well within the limits of known bacteria. In cells as large as this, green pigment should be observed if present. The complete lack of internal structure other than the fat globules is distinctly a bacterial character. The marked resemblance to *Bacterium pediculatum* Koch and Hosaeus, which is distinctly a bacterium, strengthens the opinion that the organism described by Famintzin is truly a bacterial species.

We have recently encountered an organism practically identical with *Nevskia ramosa* in morphology, save for its smaller size; the cells average about 1μ in thickness and 3 to 4μ in length. It appeared as a slimy scum upon the surface of a jar of water from the lily-pond in the University greenhouse, to which we had added a little sodium sulphide to encourage a growth of Beggiatoa. The new organism appeared intermingled with numerous Beggiatoa threads, but upon transfer to new sterilized flasks of the same water with sodium sulphide added, it has been possible to obtain a continued growth of the stalked bacterium in serial cultures with a relative decrease of the other forms. The cells contain each a number (3 to 4 on the average) of highly refractile vacuoles which resemble the fat vacuoles described by Famintzin, but these do not stain with scarlet red. The fact that the organisms are distinctly favored by the addition of sulphide to the medium makes it strongly presumptive that they are sulphur bacteria, and that the vacuoles contain sulphur in the same form in which it appears in Beggiatoa. This organism is still under investigation.

Beauverie has published an illustration of a zoögloea of *Azotobacter chroococcum* in which the cells are radially arranged about a mass of gum, each cell or pair of cells occurring at the tip of a broad lobe of gum resembling very closely the arrangement of the cells in *Nevskia ramosa*. Such a structure must, however, be exceptionally rare in Azotobacter, or it would have been recorded by others. We have examined many preparations of this organism, especially in slides negatively stained with Congo red, which shows the distribution of the slime, and have never observed such a stalked structure.

Bacterium pediculatum

This organism was found growing in the syrup of a sugar refinery as zoögloeae, macroscopically resembling those of *Leuconostoc mesenteroides*. Microscopic examination of these masses showed them to be composed of twisted, short, thick, sausage-like filaments, often branched. But heated, stained smears showed only short, fine rod forms. Examination of wet unheated material in a dilute methylene-blue solution explained the discrepancy. The rod forms were found growing at the tips of the thick thread-like structures. At the tip of each was found a single cell, with its long axis at right angles to the axis of the stalk, the stalk of "kolossalen" dimensions as compared with those of the bacterial cell. The cells multiplied by transverse fission, and each cell continuing to secrete gum on one side, the stalks became branched. The gum of which the stalks are composed is easily soluble on heating.

No dimensions are given, but from the drawing and description one gains the impression that the cells were of ordinary bacterial dimensions, say about the size of a colon bacillus. Koch and Hosaeus state that their organism shows some resemblance to certain stages of the *Bacterium vermiforme* of Marshall Ward. They were unable to obtain cultures.

Gallionella ferruginea

This is a very remarkable bacterium which has undergone many misadventures with regard to nomenclature and classification.

It was first described by Ehrenberg, who placed it in the algal genus Gaillonella of Bory de St. Vincent, but in a subsequent publication he changed the spelling to Gallionella. This name has been most widely applied by those who have studied the organism, but the species has been referred to a considerable number of different genera of algae and of bacteria by various authors (see Buchanan, 1925).

Griffith, in 1853, pointed out that the organism was not a diatom, and could not therefore be retained in the genus Gaillonella. He created the genus Didymohelix, under the mistaken impression that the microbe is composed of two intertwining filaments. Since the genus Gaillonella has been retained for certain diatoms, and since Ehrenberg offered no reason for his change of the spelling, Buchanan accepts Didymohelix as valid. But it would seem that, whether by accident or design, the change in spelling by Ehrenberg did actually create a satisfactory generic name for this organism, which is not preoccupied and is supported not only by priority but by the more important advantage of common usage.

These vagaries in nomenclature and classification may be attributed almost entirely to the complete failure of all workers previous to Cholodny to comprehend the peculiar morphology of this bacterium. It is a stalked bacterium, the stalks being composed of ferric hydroxide deposited in colloidal form from one side of the cell. As the cell grows, and the stalks increase in length, the cell slowly rotates on the stalk, which thus becomes spirally twisted. The bacterial cell is very minute as compared with the stalk, and is easily broken off. Previous workers thus observed only the inert stalks, and based their morphologic descriptions entirely upon them. It was only by observing the morphology of the organism *in situ* upon coverslips immersed in iron-bearing waters that Cholodny was enabled to solve the riddle.

The cells are kidney- or bean-shaped, 1.2 to 1.5μ in length and 0.5 to 0.6μ in thickness. They lie upon the tips of the stalks with their long axis at right angles to that of the stalk, as in the case of *Bacterium pediculatum.* They are attached to the stalk on their concave side. They multiply by transverse fission, and, following cell division, each cell continues to spin its own stalk, so that branched stalks result. The pair resulting from a cell division may move about each other, so that the two stalks become twisted together.

The stalks are, according to Cholodny, composed entirely of ferric hydroxide, because the youngest portion adjacent to the cell stains intensively with microchemical reagents for iron, and because the whole stalk completely disappears when treated with dilute hydrochloric acid. The stalks are very brittle, easily fragmenting on handling of the material. Although Cholodny described the organism as "festsitzende," no holdfasts are mentioned.

The organism is psychrophilic, growing best in cold brooks and in the springtime. It has not been cultivated upon artificial media.

Although classed by all previous authorities with the Chlamydobacteriales, it is obvious from Cholodny's studies that this classification is false. The organism is neither filamentous nor ensheathed.

Gallionella minor

This species, discovered by Cholodny, is similar to the preceding as regards the morphology of the cells, but differs in having shorter, thicker, less twisted stalks, which often become encrusted with a deposit, forming warty outgrowths that completely mask the band-like structure of the stalk. Both species may be found side by side in the same habitat.

Hyphomicrobium vulgare

First discovered by Rullmann, this organism had apparently also been observed previously by Winogradsky and by Stutzer and Hartleb, in all cases in cultures made for the purpose of isolating nitrifying bacteria. Rullmann believed that it is a nitrifying bacterium, and named it "*Nitrobacterium formaenovae.*" It grew on all ordinary media as a short rod, but upon nitrate media developed a peculiar morphology; from the rods fine hair-like processes grew out from one pole, resembling monotrichous flagella. They were, however, not flagella, since they stained readily (though faintly) with ordinary basic dyes, and the cells were non-motile. Moreover, the fine filaments were sometimes branched, especially in liquid cultures. This branching suggested a "Streptothrix," but Rullmann, who had worked extensively with soil actinomycetes, states definitely that there is no resemblance.

Nevertheless, Hartleb and Stutzer believed that the fine filaments are mycelia, and that the bacterial cells are chlamydospores from which this mycelium sprouts. They also believed it to be a nitrifying organism, and called it the "saltpeterpilz." In a later paper Stutzer and Hartleb described the microbe more fully, and gave it the name *Hyphomicrobium vulgare.* The following summary of their description is taken from the paper by Enlows: "A nitrifying (?) organism found in soil. Related to the bacteria and to the hyphomycetes. On nitrate agar, small homogeneous rods, with usually pointed ends, 0.6 to 0.8μ by 1 to 1.5μ long. Stained with phenol fuchsin a darker central body surrounded by a clear zone may be observed. Egg-shaped forms in older cultures, which send out threads, some of which show true branching. Multiplication also by transverse division. Found also in cement which they think was decomposing through the assistance of this organism."

The same or a similar organism was reported by Joshi as a nitrite-forming bacterium. We have not been able to see the original paper. The following summary has been given by Gibbs: "This organism was commonly found in two forms, one of which was chalky white in appearance, thread-like and branching like a mold, while the other form was shorter and had flagella at one pole. It would not grow in bouillon or on gelatin, and preferred magnesium carbonate." The chalky form might have been an actinomyces.

Gibbs noted a "very short stem-like growth" in many strains of his pure cultures of nitrobacter. Such growth is very noticeable when the preparation is stained by any method for staining flagella, but is seldom seen in the ordinary stained preparation.

Fred and Davenport observed in their studies of nitrobacter that "a great many of the mounts showed the cells with rather thick straight flagella-like attachments but never more than one to a cell. Occasionally these would have the appearance of typical flagella in that they were fragile and waved."

Although Stutzer and Hartleb, and also Winogradsky, had expressed doubt concerning the ability of the hyphomicrobium to oxidize nitrites, Prouty was apparently the first to look upon these stalked forms as contaminating and non-oxidizing. He states that: "In many instances the polar flagellum-like attachment which was noted when this organism was stained with cold carbol fuchsin, appeared to join a smaller deeply stained body to the bacterial cell. The length of this attachment varied in different cells. On many it was from 7 to 10μ long, whereas on others it was much shorter. In some preparations the organisms were clustered together with the flagella-like attachments radiating away from the center of the cluster of bacteria. In many instances the cells were unevenly stained, there being an area at one end of the cell, sometimes near the flagellum-like attachment and sometimes near the other end, which would not take the stain. These cells were generally oval and somewhat pointed at the ends."

The nature of *Hyphomicrobium vulgare* has recently been more extensively investigated by Boltjes, who has also concluded that it is not a nitrifying organism, but a common accompanying organism in cultures of nitrobacter. It appears regularly in such cultures because it is apparently widespread, can utilize nitrates as a source of nitrogen, and can grow, like the *Bacillus oligocarbophilus* of Beijerinck and van Delden, upon such organic matter as can be obtained from the air. When inoculated into inorganic media and supplied with air filtered through sulphuric acid and permanganate, no growth occurred. It grew, however, on a variety of organic media; a nitrate, sodium formate medium was especially favorable.

Boltjes definitely identified the filamentous attachments to the cells as stalks. They develop more strikingly in the media of lower nutrient value, and increase in length with age of the culture. The filaments cannot be readily seen in wet preparations observed by transmitted light, but become very obvious when dark-field illumination is used. In older cultures the stalks are definitely branched. The cells are oval in form. In young cultures they are motile, but when such motile cultures are examined by dark-field illumination, it is observed that the stalk is stiff and takes no part in propelling the organism. The stalks frequently show small knobs. It is typical that the bacteria often form stellate groups with the stalks turned outside and the knobs inside. The mode of reproduction could not be clearly observed, but Boltjes suspects that the process is different from that in "ordinary" bacteria. He noted strongly refractile bodies within the cells which were shifted slowly to the end, and believed that these were points where the newly developing cells are formed.

Summarizing the preceding statements, it would seem that *Hyphomicrobium vulgare* is a fairly definite bacterial species, widespread in distribution and capable of growing in extremely dilute media, with oval cells which tend to show some internal differentiation, and which grow upon stalks. Structures which we would interpret as holdfasts were observed by Prouty and by Boltjes; branching of the stalks was noted by Rullmann, Stutzer and Hartleb, and by Boltjes. A differentiation of the protoplasm into deeply stained and faintly stained portions was described by Prouty and is clearly shown in the drawing accompanying the paper of Fred and Davenport; this may be related with the refractile body observed by Boltjes.

Pasteuria ramosa

In the course of his early studies on phagocytosis, Metchnikoff discovered a microörganism parasitic in the body cavity of the water fleas, *Daphnia pulex* and *Daphnia magna*, which he referred to the bacteria and named *Pasteuria ramosa.*

It appears within the body cavity of the Daphnia as globular colonies, cauliflower-like in appearance, made up of pear-shaped cells attached to each other at their tips, to form branching and rebranching lobes. At times these colonies break up into smaller ones, and continue to separate until all of the individual cells are liberated.

The cells look like little grape seeds, and in stained preparations the individual cells may be seen to be composed of three portions; an anterior rounded body, the spore, a median thickened portion, and a posterior tapering portion. The latter part serves as the attachment of the cells to each other, and is described as a stalk, although apparently continuous with the protoplasm of the cell proper.

The cells multiply by *longitudinal* binary fission, splitting lengthwise, and thus giving rise to the branched structures. The two daughter cells remain attached at their tips. The rounded bodies which Metchnikoff describes as spores, appear in his photomicrograph as deeply stained bud-like bodies resting in cup-shaped cells, but in the drawings from unstained cells they are indicated as arising within the protoplasm and being extruded with later development. The usual spore stain differentiates them from the protoplasm in the same manner as with common spore-forming bacteria. From the published photographs of stained preparations, the individual cells appear to be 4 to 5μ in length and 1 to 2μ in thickness.

Migula dismisses this organism as being obviously a myxomycete or myxobacterium. However, Metchnikoff's description and illustrations are perfectly clear, and it is quite obvious that his organism is *not* either a slime-mold or a slime-bacterium. The longitudinal fission is a character foreign to the true bacteria as we know them. Metchnikoff ingeniously argues that fission in all planes, as in the Sarcinae, may be looked upon as a primitive character, and that in the later-developing rod-shaped organisms, some species may have retained longitudinal fission although the majority have retained only transverse fission. The single endospores are stressed by Metchnikoff as essentially bacterial characters. Unfortunately, the germination of these spores was not observed. The cells are non-motile at all stages.

Pasteuria ramosa differs markedly from the organisms which we are accustomed to consider as bacteria. It bears a strong resemblance to the blue-green algal genus, Chamaesiphon, which similarly forms pear-shaped cells attached to a substrate at the tip, and multiplies by the formation of endogenous reproductive bodies designated as "gonidia" by the algologists. We have observed several microörganisms growing upon slides immersed in Lake Alexander which show a marked resemblance to the organism of Metchnikoff, but which produce reproductive bodies apparently by budding rather than by endogenous formation. Metchnikoff clearly shows the development of spores internally. Instead of being liberated by dissolution of the cell, as in the ordinary spore-forming bacteria, they are extruded at the unattached end of the cell, a process somewhat analogous to the formation of basidiospores in the Basidiomycetes. Metchnikoff's photomicrograph shows no traces of a membrane about the spore, though such a membrane is clearly presented in his drawing.

Whether this microörganism should be considered a bacterium or not will depend upon one's definition of bacteria. In its minute size, lack of pigment, and parasitic habit of growth it resembles bacteria more than it does the blue-green algae, and it certainly cannot be included with any of the other known groups of microbes.

Bacillus flagellatus

In 1905 Mabel Jones reported a peculiar organism isolated from the city water supply and from sewage in Chicago. This bacterium grew readily on artificial culture media. It was a Gram-negative, comma-shaped organism, 1.5 to 3μ by 0.5 to 0.7μ, actively motile with a long single polar flagellum. The cells showed a marked tendency to become grouped in rosettes, with all of the flagella pointing toward an unseen center, the cells extending from the periphery, "the flagella appearing in unstained preparations of rosettes as the radial arms of a windmill to which the vanes are attached." No motility was observed in cells thus arranged in rosettes.

Nine years later Omeliansky reported finding an organism, identical in morphology with that described by Miss Jones. It grew upon plates of glycerol peptone agar which had been inoculated from a crude culture of red sulphur bacteria obtained from river water. The colonies were brownish. Slides showed the same vibrio types with single polar flagella readily visible in slides simply stained with methylene blue, and the same arrangement in rosettes. But in Omeliansky's photographs one may readily see the attachment of the so-called flagella to a common base, and small rounded expansions at the tips of the "flagella" of cells which are not in rosettes, that look like the holdfasts of some of the stalked bacteria which we have observed. Omeliansky makes no mention of motility. Subcultures failed to grow.

The strong resemblance between the organisms shown in illustrations accompanying both of the above papers and the stalked bacteria which we have observed upon immersed slides leads us to believe that the structures described as flagella were in reality stalks. This is supported by the fact that they stained so readily without any mordanting. Opposed to this view is the motility definitely recorded by Miss Jones.

Vibriothrix tonsillaris

This organism was first described by Davis and studied further by Davis and Pilot, and by Davis and Hall. It is found in the crypts of human tonsils forming small greyish or yellowish granules. Similar granules have also been found in dental root abscesses and in sputum from cases of bronchiectasis. They have been repeatedly mistaken for granules of actinomycetes, with which, however, they are not related.

The granules are made up of central shafts of "mycelial filaments" about which radiate bacillary forms, resembling closely the fusiform bacilli of the mouth. Associated with the bacillary forms are cocci and spirilla, which, however, may be other organisms mechanically adhering to the granules.

These granules have been cultivated by Tunnicliff, and further studied in cultures by Tunnicliff and Jackson. They grow anaerobically in ascites agar, in the form of granules similar to those occurring in the tonsils. A number of coccoid and spiral forms were also found in cultures, but it is not certain that these were pure cultures.

The essential structure is a central filament, or more commonly a bundle of filaments, to which the bacteria cells are attached. Both Davis and Tunnicliff have intimated that the relationship is like that in a mold, that the central filament is mycelium and that the bacillary bodies are conidia. We suggest, however, that the filaments are *stalks* secreted by the bacteria, and that the granules are merely colonies of stalked bacteria.

This opinion was arrived at first by our observation of distinctly stalked fusiform bacilli on slides submerged in lake water. The bacillary bodies as described by Davis are indistinguishable from fusiform bacilli, both in form and in staining reactions. Tunnicliff noted that the ends of the cells in cultures were rounded or square rather than pointed, but did find the characteristic granules staining red with Giemsa stain which are present in fusiform bacilli.

The central filaments are very fine, apparently unbranched, and stain faintly as compared with the bacillary forms. They are homogeneous in structure. These characters resemble closely those of the stalks of the bacteria we have studied, not an organized protoplasmic mycelium.

This organism was referred to the genus Vibriothrix of Castellani and Chalmers by Tunnicliff and Jackson at the suggestion of Castellani. The genus has been so vaguely described, however, that it might include almost any pleomorphic bacterium.

STALKED BACTERIA FROM LAKE ALEXANDER

A variety of stalked bacteria which we have observed on slides immersed in Lake Alexander are illustrated in plate 2. It will be more convenient to describe them by reference to the various figures of this plate. All of these figures have been drawn accurately from projected photomicrographs. They are illustrated in this manner to conserve space. Photomicrographs of four types are, however, presented in plate 3. Although all of the types here reported are described from Lake Alexander, we have found many of them to be ubiquitous, and anyone may readily confirm our observations by examining slides immersed for a few days in stagnant water, or even in running tap water.

Figure 1 shows a stalked vibrio which appears very similar to the illustrations published by Miss Jones and by Omeliansky. It is a trifle larger than the dimensions given by Miss Jones. The cells are always distinctly curved, with rounded ends. Multiplication occurs by transverse binary fission. The stalks are very slender, and often show a distinct button-like expansion at the end, which we consider to be a holdfast. It is probable that the outermost cell is set free after cell division, and either swims or floats away until a new substrate is encountered, when it proceeds to secrete a stalk. All examples studied by Gram's stain have failed to retain the stain.

This is probably the most common type encountered. It has been found both in the open lake and in the shallow, weed-choked bays, and at all depths up to 8 meters. It is, however, a little more abundant in the shallower waters. It has been observed at all seasons except when the lake was frozen, at which time no observations have been made.

Figure 2 shows a small, straight rod form, with short, slender stalks and very prominent, button-like holdfasts. It is Gram-negative, and multiplies by transverse binary fission. It is quite rare, and has been found on only a few slides from shallow water. When it does occur, however, large areas of the slide are covered by the rather widely scattered organisms.

Figure 3 illustrates a large, relatively straight rod form with rounded ends. It is Gram-negative. It is also relatively rare, occurring usually in groups of three or four individuals. Division has not been observed.

Figures 4, 5, and 6 show fusiform types. These are abundant and vary considerably in size. We are of the impression that they tend to group about three modal sizes, as illustrated, and that these represent three distinct species, because they often occur in rather extensive microcolonies in which all of the cells are of nearly the same size.

All of the fusiform types which we have observed by Gram's stain were negative. Multiplication is by transverse binary fission. The cells are pointed at both ends. The protoplasm stains more deeply in the center of the cell, and merges into the slender stalk. We have not yet obtained satisfactory Giemsa stains of these organisms, so we cannot say for certain whether they exhibit granules of the type shown by the fusiform bacilli of the human mouth. The stalks are slender in the smaller species, rather coarse in the largest one. Holdfasts have been observed in all.

After cell division the outermost cell develops a long slender tip, and we have in a few instances found a pair of cells with a stalk and holdfast at both extremities of the pair. We believe that in these species the outermost cell develops its stalk and becomes anchored to the substrate before the two are finally separated, in this way extending rapidly over the substrate. This view is supported by finding the fusiform types commonly in very extensive microcolonies, extending over many oil immersion fields.

The fusiform species have been found frequently in all of the lake habitats which we have investigated, and in the open lake at all depths up to 13 meters.

Figure 7 shows a puzzling form, not very frequent in occurrence, but rather widely distributed. The cells are swollen, irregular in form, and often triangular. The larger forms stain faintly in the center, as though they were ballooned by a vacuole. We are inclined to the opinion that they are involution forms of the vibrio shown in figure 1. But this opinion is opposed by finding them in one instance fairly abundant on a slide which was immersed in the lake for only twenty-four hours.

The species considered so far all show a certain degree of resemblance. They are all essentially bacillary in form, and show definite transverse fission (save in the types shown in figure 3, of which only a small number of individuals have been examined; and in figure 7, which are probably involuntionary). While occurring in extensive microcolonies, they do not tend to form clusters, and the stalks are not branched. Although both Miss Jones and Omeliansky observed radiating clusters in their cultures, we are of the opinion that the organisms just described are very similar to those reported by these authors, and that the formation of radiating clusters may be a peculiarity of artificial cultures. When growing close together on agar, they may well become attached to each other rather than to the agar. In any case, our stalked vibrio (figure 1, plate 2) bears a striking resemblance to the organism illustrated by Omeliansky (figure 5, plate 1) which he named *Bacillus flagellatus.*

The remaining forms which we have observed differ markedly from the above mentioned types, and resemble in many respects the organism described by Metchnikoff as *Pasteuria ramosa.*

Figure 8 represents a fairly common water organism. It is a short, plump rod, not stalked, but showing in common with the stalked bacteria a polar differentiation of the cells. There is a base and a tip. At the base an amorphous material, probably gum, is secreted to fasten the cells to the substrate. This material often extends from the cell in a fan-like manner. The Gram reaction is variable, both positive and negative individuals appearing in the same field. Multiplication apparently is entirely by budding, or by a process intermediary between budding and fission. The buds present the form of the parent cell, but are smaller both in length and in thickness.

Figure 9 shows a somewhat similar organism without a definite holdfast. The cells are swollen at one end, and stain deeply at this end, faintly at the pointed end which is probably attached to the glass in some manner. Occasionally two cells are arranged in a V form, possibly indicating recent longitudinal fission, but this is not certain. Multiplication by buds is at least the more frequent, if not the sole, mode of multipliction. Young buds are spherical, but become oval or even rod-shaped before they are liberated.

Figure 10 illustrates a species which is more common than the two preceding, and which differs from them in occurring distinctly in rosettes. The cells are elongated and piriform in shape, and are attached to each other at their tips. They usually stain solidly. Often a mass of cementing material may be seen about the common base of a rosette, and occasional isolated cells show a distinct short stalk and holdfast. The rosettes very obviously arise by longitudinal fission. In addition, reproduction by budding is commonly observed. The buds are oval to rod-shaped in form. Save for this last character, the organism is almost identical in morphology with the *Pasteuria ramosa* of Metchnikoff.

This species has been found with equal abundance in all of the habitats studied in Lake Alexander. In the open lake it occurs at all depths up to 13 meters.

Figure 11 shows a small coccoid form which is not definitely a stalked bacterium, yet shows some resemblances to the type shown in figure 10, and may possibly be a growth form of the type illustrated in figure 14. The cells occur in clusters cemented together by amorphous material, and tend to be arranged in rosettes. The outermost cells often show minute spherical buds, and this is the only mode of multiplication that we have observed. These forms are not abundant.

Figure 12 represents another organism of very doubtful nature, which is offered here merely for comparison with the preceding. It is a large spherical organism multiplying by budding. It may possibly be a small yeast, but it would be surprising to find yeasts growing attached to a firm substrate in water.

Figure 13 presents two examples of a group of microbes which show a variety of forms, and which will require further study before their specific characters may be delineated. They occur as single globular, oval or pear-shaped cells upon a stalk which is fastened to a substrate. There is an oval type whose cells are often vacuolated, which is especially common. Multiplication is apparently entirely by budding.

Figure 14 illustrates one of the most striking of the stalked microörganisms which we have encountered. The cells are globular in form, attached to long slender stalks which radiate from a common center. Multiplication is by budding, and the buds are also globular. The smaller cells stain solidly, but the larger cells which are budding show a differentiation of the protoplasm, the outer part staining deeply while that part of the cell to which the stalk is attached stains more faintly. As many as 8 stalks have been observed attached to a common holdfast. Usually they are attached directly to the glass, occasionally to algae or other organisms or to some amorphous débris. The young, solid-staining cells are Gram-positive, but budding individuals are usually Gram-negative.

We believe that the characteristic growth of this organism in whorls may be best explained by assuming that when the buds germinate they first undergo a multiple fission, perhaps producing clusters of cells as shown in figure 11, and that then, from these clusters, the individual cells secrete stalks, which thus radiate from a common holdfast.

This organism is distinctly psychrophilic. It has been found only in the open lake, where temperatures do not exceed 23°C., never closer to shore than the 2-meter contour. It has been found constantly in several different stations in the open lake, at all depths up to 13 meters. It occurs more abundantly in the fall months than in the summer.

Figure 15 presents a type which differs from the preceding only in the thick, tapering stalks. We cannot determine whether this is a separate species. In some clusters both thick stalks and slender stalks occur. Both types of stalks may be formed by the same species, or a slender-stalked species may become attached to a thick-stalked one. Both types may be found on the same slide.

Figures 16 and 17 illustrate another species resembling the preceding. The cells are larger and pear-shaped. The same peculiar difference in the staining of budding and non-budding cells is observed. Mature buds are themselves pear-shaped, and may also show a differential staining. This organism is also peculiar in that we have never observed it attached directly to the glass. It is always attached to a mass of amorphous débris adherent to the glass.

This pear-shaped organism is not nearly so abundant as the globular form illustrated in figure 14. It is also found only in the open lake, and has been observed at depths up to 7 meters. Curiously, it was much more abundant in 1934 than in 1933.

In all of the microörganisms illustrated in figures 8 to 17 inclusive we have referred to multiplication by budding as a common and striking character. Concerning the nature of these buds we are still a little in doubt, because they resemble to some extent the bodies described by Metchnikoff as spores in *Pasteuria ramosa.* We have attempted to demonstrate a membrane surrounding the bud by various staining methods, especially by Fontana's silver impregnation, without success. Conversely, by the use of Congo-red negative staining we have clearly demonstrated that there is no membrane; the stain penetrates readily between the bud and the parent cell, leaving only a small band of attachment. Moreover, we see all stages of development in the buds, from minute globular bodies to masses nearly as large as the parent cell. We are forced to conclude therefore that either Metchnikoff was dealing with an entirely different sort of a microbe, or that his observations were erroneous. The strong resemblance between the organism illustrated in our figure 10 and Metchnikoff's *Pasteuria ramosa* inclines us to believe that we are studying microbes of the same general character.

There is a strong resemblance between the organisms illustrated in figures 13 to 17 (plate 2) and the *Hyphomicrobium vulgare* of Stutzer and Hartleb. In both, there is a tendency for the cells to assume a globular or ovoid form, and to show a differential staining in the protoplasm. In both, there is a tendency to dichotomous branching of the stalks, which may be explained only upon the basis of longitudinal fisssion. The photomicrograph which Rullmann published might well pass for an illustration of the type which we have illustrated in figure 14, save for the absence of buds. In fact, the complete absence of any mention of budding by the various authors who have studied hyphomicrobium is all that prevents us from concluding that our organisms are identical or closely related. Until the mode of reproduction of hyphomicrobium has been determined, we shall be unable to state definitely to what degree it is related to the forms which we have described.

Figure 18 is another organism of very doubtful nature, in fact we are not certain that it is a microörganism. It is offered here merely for record, since it may possibly with further study prove to be a stalked bacterium.

The material appears in tangled masses much resembling windrows of hay. The portion illustrated is less dense than usual. These masses are composed of fine, faintly-stained filaments, which do not appear to branch. At either extremity of the filament is a deeply stained spherical body, one of which usually appears a little larger than the other. It is possible that one is a cell and the other a holdfast, but both are close to the limits of resolution of the microscope, and nothing may be said with certainty.

CULTIVATION

No extensive experiments have been carried on with a view to the isolation of these organisms in pure culture and their study by standard methods, since we have been engaged primarily in a study of the bacteria by purely microscopic methods in their natural habitat. A few crude cultures have, however, been obtained.

Samples of water from Lake Alexander were collected in the summer of 1934 and brought to the laboratory in Minneapolis. They were inoculated into a variety of liquid media. A growth of stalked bacteria was obtained in two of these, one a mineral solution containing precipitated cellulose, with ammonium salts as the nitrogen source; the other a solution of $MgSO_4$ and K_2HPO_4 in tap water, to which bits of exoskeleton of marine crabs were added. The types shown in figures 1, 4, and 13 (plate 2) appeared in both of these media after 48 hours incubation, but together with a great many other types of bacteria, especially vibrios. It was possible to carry them through separate transfers, and some are still growing after five months. But it has been impossible to obtain a growth upon agar, and therefore we have obtained no pure cultures as yet. Further attempts are in progress. These cultures were incubated at room temperature in the dark.

TAXONOMY

A consideration of the classification of these microbes at once raises the question whether they may be considered as bacteria or not. Some of them are obviously bacteria, but others, such as the pear-shaped and globular forms multiplying by longitudinal fission and by budding, seem at first glance to be far removed from the organisms ordinarily thought of as bacteria.

We offer as essentially bacterial characters, the following: *1. Size.* The largest observed is but a little over 2 μ in diameter. They might be very small forms of algae or protozoa, but in that case one would expect to observe forms intermediary between these minute bacteria-like bodies and the larger microbic species. This has not been observed. *2. Structure.* While in some of the larger species there has been observed a differentiation of the protoplasm into a deeply stained and faintly stained portion, this is in general true only of budding cells, and is not a constant characteristic. There is, therefore, no differentiation of the protoplasm, such as would be expected in cells of algae or protozoa. Nothing resembling a nucleus or central body has been observed. *3. Nutrition.* The fact that many of the forms described have been uniformly distributed in depths up to 13 meters indicates that they are not photosynthetic. The algae which grow upon the same slides show a marked gradation from the surface to the bottom, obviously correlated with light penetration, and are relatively rare at depths below 3 or 4 meters. Further, the fact that some of these forms have been grown in the dark in crude cultures containing organic matter indicates that they are not photosynthetic, but suggests that they are heterotrophic. *Gallionella ferruginea* is usually considered autotrophic, though this is denied by some authorities. It is obvious that none of these stalked bacteria are capable of ingesting solid food like the protozoa. It seems highly probable, therefore, that as a group they are heterotrophic, i.e., saprophytes. Metchnikoff's organism was parasitic.

The best argument for including these microörganisms with the bacteria is the fact that they cannot be included in any other group. Their minute size, structureless cells, and saprophytic or parasitic modes of life indicate that they belong with the bacteria rather than with the algae or protozoa, the only other possible places for them. Our conceptions of the limitations of the class "Schizomycetes" are still rather vague. There is nothing in Bergey's definition of the class which would exclude the organisms here considered.

Assuming that they may be properly included with the bacteria, it remains to determine how they should be grouped within this class. The taxonomic value of morphologic characters increases with decreasing diversity of form of the organisms to be classified. It seems to us that with microbes so slightly diversified as the bacteria, the occurrence of structures so unusual as stalks is sufficient to justify the recognition of such bacteria as a distinct order, certainly sufficient to distinguish them from the Eubacteriales. They are obviously not actinomycetes or spirochaetes. We have already pointed out how they differ from the myxobacteria. They resemble the Chlamydobacteriales in presenting an axial differentiation of the cells into basal and apical portions, and in their aquatic habitat, and in the formation of holdfasts; but differ in failing to form filamentous chains of cells, and sheaths. While one species may possibly be concerned with the oxidation of sulphur, this is not true of the group as a whole.

It may be questioned whether the grouping of all stalked bacteria into a separate order is a "natural" arrangement, i.e., whether these organisms are actually phylogenetically related. Concerning this, we do not have enough information to venture an opinion, but the same is equally true of the other orders of bacteria as now defined. Such a classification is, however, of some practical value. It brings together in some order a variety of little known forms whose descriptions are widely scattered in the literature, and which so far have had no place in the recognized classifications of bacteria.

We therefore propose the creation of a new order, defined as follows:

Caulobacteriales. Bacteria growing characteristically upon stalks. The cells are asymmetrical in that gum, ferric hydroxide, or other material, is secreted from one side or from one end to form the stalk. Multiplying typically by transverse binary fission; in one family by budding and longitudinal fission. In some species stalks may be very short or absent, the cells connected directly to the substrate or to each other by holdfasts. Cells occur singly or in pairs, never in chains or filaments; not ensheathed. Typically aquatic in habitat; some may be parasitic in animals.

The order is naturally subdivided into four divisions by major differences in morphology. We recognize these divisions as families.

I. **Nevskiaceae.** Stalked bacteria, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks lobose, dichotomously branched, composed of gum. Multiplication of cells by transverse binary fission. Growing in zoögloea-like masses in water or in sugar vats.

One genus, *Nevskia* Famintzin. Type species, *Nevskia ramosa* Famintzin. A second species is *Nevskia pediculata* (nov comb.) (Koch and Hosaeus). H. and J.

II. **Gallionellaceae.** Stalked bacteria, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks are slender, twisted bands, dichotomously branched, composed of ferric hydroxide. Multiplication of cells by transverse binary fission. Growing in iron-bearing waters.

One genus, *Gallionella* Ehrenberg. Type species, *Gallionella ferruginea* Ehrenberg (more accurately described by Cholodny). A second species is *Gallionella minor* Cholodny.

III. **Caulobacteriaceae.** Stalked bacteria, the long axis of the elongated cells coinciding with the axis of the stalk. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, unbranched. Multiplication of cells by transervse-binary fission. The outermost cell of a pair may form a stalk before cell division is complete. Periphytic, growing upon submerged surfaces.

One genus, *Caulobacter* (nov. gen.).

If it could be established beyond question that the organism described by Omeliansky as *Bacillus flagellatus* was actually stalked rather than flagellated, this could readily be taken as the type species. Since this cannot be done, we propose as the type species the organism illustrated in figure 1, plate 2, as:

Caulobacter vibrioides (n.s.). Cells elongated, curved, with rounded ends, 0.7 to 1.2 × 2–5 microns.

The types illustrated in figures 2 to 7 inclusive (plate 2) will also fall in this genus, but specific names will not be given until they are studied further.

IV. **Pasteuriaceae.**[2] Stalked bacteria, the long axis of elongated cells coinciding with the axis of the stalk. Stalks may be very short, in some species lacking, but when present are usually very fine and at times arranged in whorls attached to a common holdfast. Cells multiplying by longitudinal fission or by budding, or both, spherical or pear-shaped in most species. Mostly periphytic, one species parasitic.

Genus 1. *Pasteuria* Metchnikoff. Stalks short or absent, the cells attached directly to the substrate or to each other, often with distinct holdfasts. Type species, *Pasteuria ramosa* Metchnikoff.

The organisms which we have illustrated in figures 8 to 10 inclusive (plate 2) will fall in this genus. We withhold specific names until they have been studied further.

Genus 2. *Blastocaulis* (nov. gen.). Stalks long and slender, often arising in whorls from a common holdfast.

We take as the type species the organism illustrated in figure 14 (plate 2), which we name:

Blastocaulis sphaerica (n.s.). Cells spherical, multiplying characteristically by budding.

The organisms shown in figures 13, 15, 16, and 17 also belong in this genus, but specific names are withheld until further studies have been completed.

[2] A family of "Pasteuriacées" was proposed by Laurent to include the organism of Metchnikoff and the root-nodule bacteria of legumes. It is now, however, obvious that the branched bacteroids of *Rhizobium leguminosarum* have nothing in common with these stalked bacteria. We retain the family name, but redefine it.

The proposed classification takes care of the well-defined types. It does not include the organisms illustrated in our figures 7, 11, 12, and 18 (plate 2) nor the *Vibriothrix tonsillaris* Tunnicliff and Jackson, which are as yet too poorly understood for classification. We believe that *Hyphomicrobium vulgare* will eventually prove to be identical with one of the species of our genus Blastocaulis, but we cannot definitely place it in our classification until its mode of reproduction has been discovered.

SUMMARY

A study of periphytic bacteria upon glass slides immersed in freshwater habitats shows the general occurrence of a group of bacteria hitherto almost unknown, which secrete stalks by which they are attached to a firm substrate. A search of the older bacteriological literature has shown that similar types have been observed before, and has also revealed certain other kinds of stalked bacteria which we have not observed. It is proposed to include all of these stalked bacteria in a new order of Schizomycetes, the **Caulobacteriales.** It is proposed to subdivide this order into four families and five genera according to the following key:

A. Long axis of cell transversed to long axis of stalk; stalks dichotomously branched.
  I. Stalks lobose, composed of gum, forming zoögloea-like colonies.
    Family — **Nevskiaceae**
    Genus — *Nevskia*
  II. Stalks are twisted bands of ferric hydroxide
    Family — **Gallionellaceae**
    Genus — *Gallionella*
B. Long axis of cell coincides with axis of stalk.
  I. Reproducing by transverse fission, stalks unbranched
    Family — **Caulobacteriaceae**
    Genus — **Caulobacter**
  II. Reproducing by longitudinal fission and by budding; stalks often branched in whorls.
    Family — Pasteuriaceae
    a. Stalks very short or lacking, cells sessile
      Genus — *Pasteuria*
    b. Stalks long and slender
      Genus — **Blastocaulis**

The scientific names in this summary, which are set in bold-faced type are new.

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PLATES

PLATE 1

Stalked Bacteria from the Literature

All figures have been redrawn from the original publications

Fig. 1. *Nevskia ramosa* Famintzin. (*a*) Zoögloeal masses, showing bacteria at the tips of the stalks. (*b*) Bacterial cells showing prominent fat globules.

Fig. 2. *Bacterium pediculatum* Koch and Hosaeus.

Fig. 3. *Pasteuria ramosa* Metchnikoff. (*a*) From photomicrographs of cells and clusters of cells, stained. (*b*) From photomicrograph of a cell with a spore. (*c*) From drawings of colonies, unstained. (*d*) Vegetative cells, unstained. (*e*) To *l*, stages in spore formation and liberation.

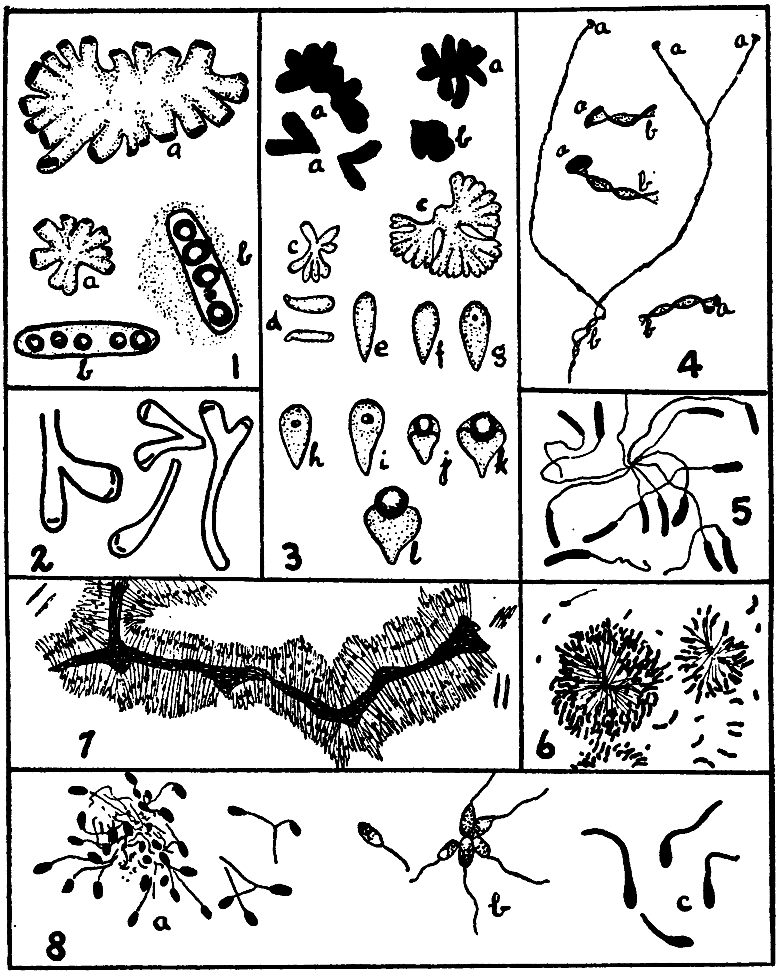
Fig. 4. *Gallionella ferruginea* Ehrenberg, after Cholodny. (*a*) Bacterial cells. (*b*) Stalks of ferric hydroxide.

Fig. 5. *Bacillus flagellatus* Omelianski.

Fig. 6. The organism of Mabel Jones.

Fig. 7. *Vibriothrix tonsillaris*, Tunnicliff and Jackson.

Fig. 8. *Hyphomicrobium vulgare* Stutzer and Hartleb. (*a*) After Rullmann. (*b*) After Fred and Davenport. (*c*) After Boltjes; Zettnow's stain.

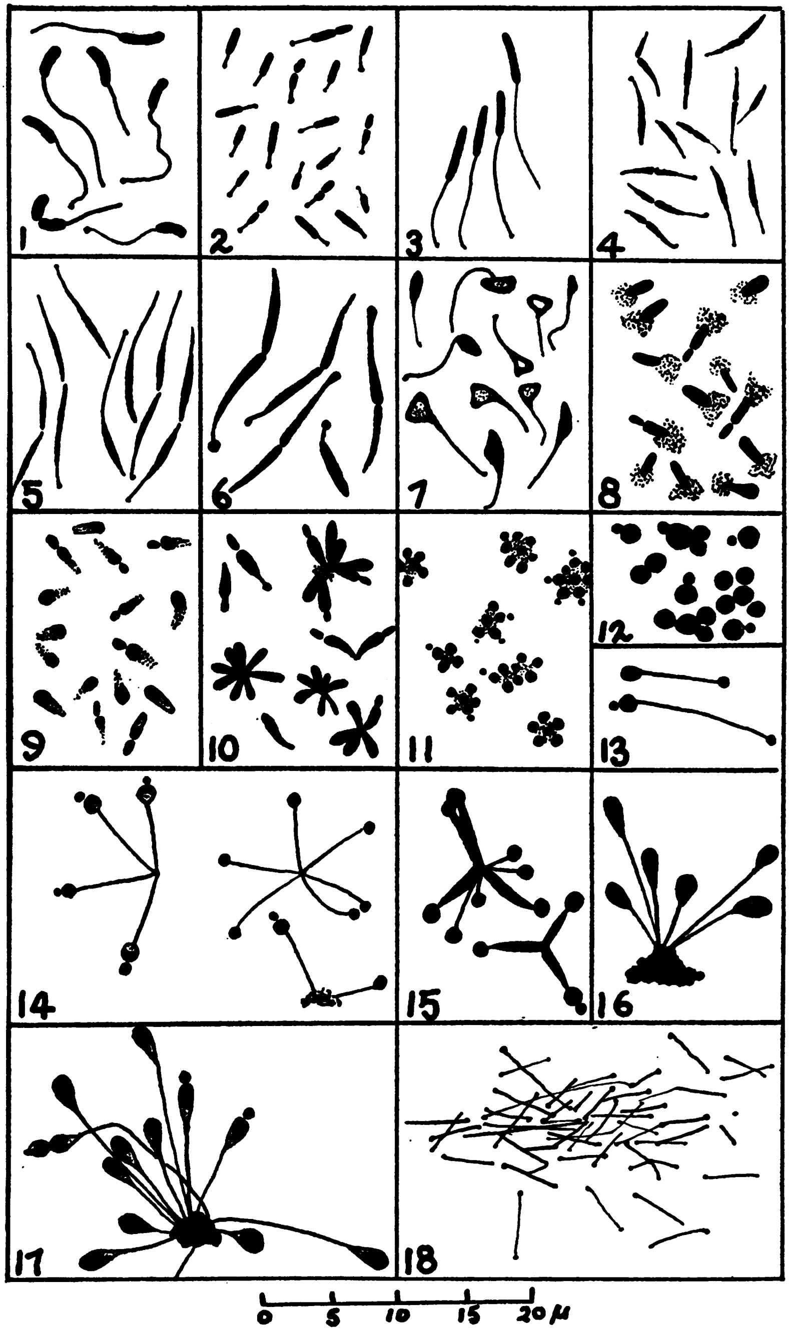


(Arthur T. Henrici and Delia E. Johnson: Studies of Freshwater Bacteria)

PLATE 2

STALKED BACTERIA FROM LAKE ALEXANDER

Explained in the text



(Arthur T. Henrici and Delia E. Johnson: Studies of Freshwater Bacteria)

PLATE 3

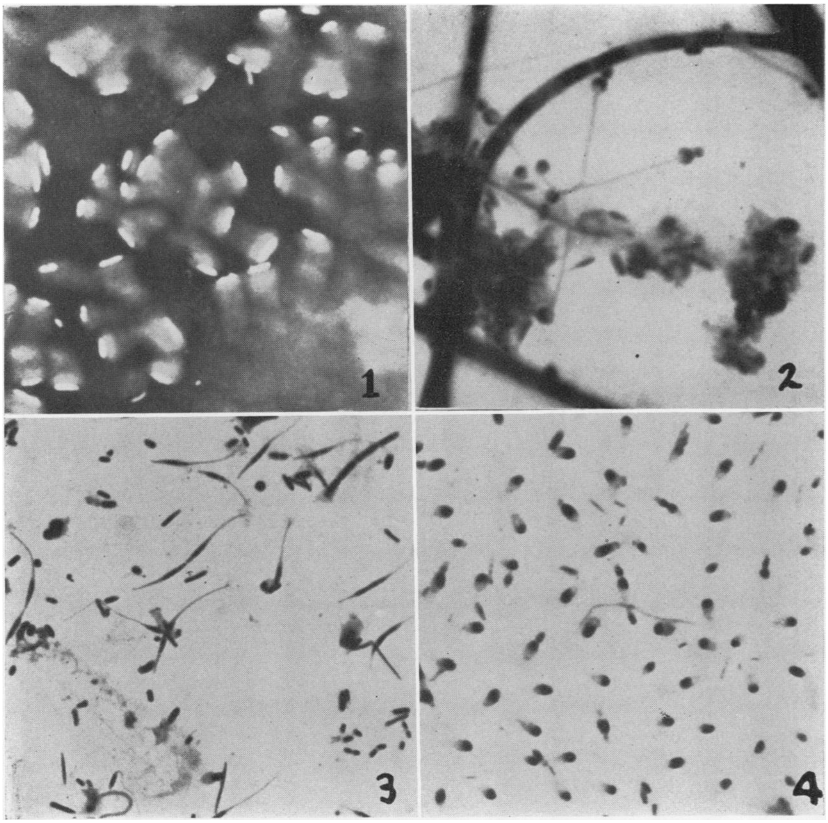
Photomicrographs of Stalked Bacteria

Fig. 1. *Nevskia sp.*, cultivated in water with sulphide; negatively stained with Congo red.

Fig. 2. *Blastocaulis sphaerica,* showing branched stalks and budding. The coarse filaments are *Cladothrix dichotoma.*

Fig. 3. *Caulobacter sp.* This is the larger fusiform type; holdfasts may be seen at the tips of some of the stalks.

Fig. 4. *Pasteuria sp.* The differential staining of the protoplast, and reproduction by budding are shown.



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