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Prosthecomicrobium and *Ancalomicrobium*: New Prosthecate Freshwater Bacteria

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Direct microscopic examination of natural freshwater samples reveals a variety of small microorganisms having elaborate cellular appendages. Several strains have been isolated from crude cultures containing low concentrations of organic nutrients. All of the isolates are procaryotic. They are aerobic chemoorganotrophs that require vitamins for growth. Because they cannot be assigned to any of the existing bacterial genera, two new genera are proposed: *Ancalomicrobium* for organisms which have several long appendages and which reproduce by budding; *Prosthecomicrobium* for organisms which have many short appendages tapering toward a blunt tip and which reproduce by binary fission. Gas vacuoles have been found in strains of each genus. The term *prostheca* is proposed for the rigid appendages of procaryotic cells bounded by the cell wall, and is defined to include the structures on these new bacteria, as well as the stalks of the caulobacters and the hyphae of the hyphomicrobia.

A variety of bacteria with unusual shapes and life cycles have been discovered in fresh and salt waters. Perhaps the most remarkable of these forms are those with cellular appendages (extensions of the cell wall containing cytoplasm). In this group are the caulobacters, whose rod- to vibrio-shaped cells have an appendage termed a stalk located in a polar (*Caulobacter*) or subpolar (*Asticcacaulis*) position. The division cycle of the caulobacters differs markedly from the typical eubacterium (24, 34). Stalked cells in these genera divide by transverse fission to produce two morphologically distinct cells. One daughter cell retains the stalk, whereas the other is a flagellated swarmer cell with no stalk. After it separates from the stalked cell, the swarmer cell does not divide until it, too, has developed a stalk. The caulobacters attach to cells and nonliving substrates by means of a holdfast, an adhesive material located at the pole of the stalked end of the cell.

The hyphomicrobia, *Hyphomicrobium* and its photosynthetic counterpart, *Rhodomicrobium*, also have cellular appendages. These organisms are rod- to pear-shaped with appendages, termed hyphae, that extend only from the polar regions of the cells. The division cycle is even more complex than that of the caulobacters (19; P. Hirsch, *personal communication*). Reproduction occurs by the formation of a bud either directly from the mother cell or at the distal tip of a hyphal filament, which may be branched. These buds are flagellated and normally detach from the mother cell or hypha.

Another similar genus is *Pedomicrobium*, which was recently described by Aristovskaya (2). The principal difference between this budding organism and *Hyphomicrobium* is that the hyphae extend in all directions from the cell; they are not restricted to a polar location.

The genus *Planctomyces*, first described in 1924 by Gimesi (9), also belongs to this group. The pear- to sphere-shaped cell has an appendage extending from one pole. Buds are formed at the opposite pole of the cell. Typically, the cells are seen in rosette clusters connected together at the distal tips of the appendages. Henrici and Johnson (12), unaware of the previous publication, described this genus as *Blastocaulis*. No strains have been isolated.

In June 1964, with the intent of studying these unusual bacteria with appendages, freshwater samples were collected and enrichment cultures were prepared with the aid of Richard Martucci. During examination of these cultures, objects of bacterial size having multiple appendages were observed. On the assumption that these were previously unknown microorganisms, attempts were made to observe them by electron microscopy and to isolate them from crude cultures.

MATERIALS AND METHODS

Techniques of microscopic examination. Strain 4a was grown in slide culture in the 0.01% YAmG

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medium (Table 1) solidified with 0.4% Ionagar (Consolidated Laboratories). Approximately 1 ml of this liquefied medium was poured onto a glass slide and was allowed to solidify and dry slightly. A drop of a 12- to 24-hr slant culture was spread over the surface of a clean coverslip which was then inverted and placed on the agar, the cells lying between the agar and the coverslip. The agar extending beyond the coverslip was removed, and the excess moisture was absorbed with bibulous paper before the coverslip was sealed to the slide with vaspar. Fields that contained one or a few cells were found, and photographs were taken at about 1.5-hr intervals on Adox KB-14, 35 mm film, with a Zeiss photomicroscope.

Freshwater samples were collected aseptically and were examined directly with the electron microscope. The particulate fraction of each sample was sedimented by centrifugation at 13,000 × *g* for 20 min. The supernatant fraction was decanted, and the pellet was resuspended to give a 50- to 100-fold concentration of the original sample. Droplets of this concentrate were placed on a Formvar-coated, 200-mesh, copper grid, and, after about 1 min, excess moisture was absorbed with filter paper. A drop of 1% phosphotungstic acid (PTA), *p*H 7.2, was added to the preparation and was immediately absorbed with filter paper. The same procedure was followed when observing whole cells from pure cultures.

For sections, the fixation procedure of Ryter and Kellenberger (27) was followed, except that strains 3a and 4a were washed in 1:10 dilutions of the Kellenberger buffer. The agar blocks were dehydrated with ethyl alcohol, transferred to propylene oxide, and embedded in a plastic mixture [11.5% Epon 812, 34.7% Araldite 6005, 52.1% dodecenyl succinic anhydride (DDSA), and 1.7% dibutyl phthalate] containing benzyldimethylamine accelerator. The blocks were then polymerized for 2 days at 60 to 80 C.

Thin sections were cut with a diamond knife in a Porter-Blum MT-1 ultramicrotome. They were poststained for 20 min in uranyl acetate, followed by 5 min in 0.1% lead citrate in 0.1 N sodium hydroxide (36).

All preparations were observed in an RCA EMU-3E or -3G electron microscope with a 50-μ aperture. The accelerating voltage was 50 kv.

Media. All media contained 20 ml per liter of the vitamin-free mineral salts solution (MS), *p*H 7.2, of Hutner, as modified by Cohen-Bazire et al. (6).

The vitamin solution contained (per liter): biotin, 2 mg; folic acid, 2 mg; pyridoxine·HCl, 10 mg; riboflavine, 5 mg; thiamine·HCl, 5 mg; nicotinamide, 5 mg; calcium pantothenate, 5 mg; B_{12}, 0.1 mg; and *p*-aminobenzoic acid, 5 mg. A sample of this solution (10 ml), sterilized by autoclaving or membrane filtration, was added to each liter of all media except GY and YE.

Table 1 lists the composition of the media used.

Methods of enrichment and isolation. Natural freshwater samples, always collected aseptically at a depth of about 1 ft, were used on the same day as an inoculum (1 ml per 100 ml of enrichment medium) or as a mineral base (100 ml per 100 ml of enrichment medium) in 250-ml Erlenmeyer flasks. The *Caulobacter* enrichment procedure of Houwink (13), in which peptone is added to the water sample at a final concentration of 0.01%, was used routinely. Additionally, the peptone was added to a mineral salts solution (2 ml of MS with 98 ml of distilled water, MS + DW), autoclaved, and inoculated with 1 ml of the water sample. Other nitrogen and carbon sources were also used at low concentrations. Cultures were incubated in the laboratory at room temperature.

A slide culture technique developed by R. Martucci was used to screen various media in order to determine which would support growth of the new organisms. A drop of the enrichment culture was mixed with a drop of the test medium, and the mixture was spread over the surface of the cover slip with another cover slip. The cover slip was then inverted and placed on a slide containing two support cover slips so that the agar medium was suspended over an air space. A water droplet was placed beneath each support cover slip to insure a high humidity. All edges were sealed with vaspar. The arrangement is illustrated in Fig. 1. The slide was then scanned to locate several fields containing the organism of interest. A drawing was made of each field, and its position was recorded from the stage graduations. Daily examination was sufficient to determine if growth had occurred. This technique took advantage of the distinctive cellular structure of forms with appendages, which permits location of the organism prior to its growth.

Several strains were isolated by passing a portion of an enrichment culture through a column containing glass beads (*see* Results). The glass chromatographic column (internal diameter, 1 cm) was filled to a depth of 2 inches with autoclaved Ballotini beads (approximately 0.2 mm in diameter). The column was washed with approximately 100 ml of hot, freshly autoclaved MS + DW and was allowed to cool to room temperature. A sample of the enrichment culture (1 ml) was carefully added to the top of the beads, and 1 ml of fluid was drained from the bottom so that the upper meniscus coincided with the top of the beads. Cool, sterile solution was added carefully to the column, and single effluent drops were collected. The first drop was added to a liquid medium of the same composition as the enrichment culture; the second was spread on the same medium (solidified with 1.5% Difco agar) in a petri dish. This procedure was repeated; i.e., alternate drops from the column were inoculated into a liquid medium or spread on a solid medium until about 30 drops had been tested. After growth for about 3 weeks at room temperature, the liquid cultures were examined macroscopically and microscopically for growth. If a liquid culture contained the desired organism, then the colonies on the corresponding petri plate were examined individually with a microscope.

Methods of bacteriological characterization. Growth yields were determined by optical density measurements with a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) at 600 mμ. The relationship between the dry weight of strain 9b and its corresponding optical density remained linear to the highest optical density measured, 0.440.

For the determination of vitamin requirements,

TABLE 1. *Composition of media*

Medium	Ingredients[a]							
	Ammonium sulfate	Phosphate	Glucose	L-Fucose	L-Rhamnose	Peptone	Yeast extract	Casamino Acids
	%	M	%	%	%	%	%	%
GY			0.01–0.05[b]				0.1	
PYGV			0.025			0.025	0.025	
PYFR				0.012	0.012	0.025	0.025	
YE[c]	0.1	0.04	0.1				0.5	
GCA			0.025–0.05[b]					0.1
GCAAm	0.025		0.02					0.01
YAmG	0.025		0.025				0.01–0.025[b]	

[a] At the final concentration in basal medium (MS and vitamins).
[b] Actual concentration noted before medium in text.
[c] Medium originally described by Stanier et al. (29).

all organisms were grown on a Vitamin Free Casamino Acids medium (Difco GCA or GCAAm). The preliminary determination of requirements was made by a single deletion of each of the nine vitamins of the vitamin solution. Yields were determined after 1 week on a shaker at 30 C. If the optical density of a culture was significantly lower than that of the control containing all nine vitamins, then a requirement was suspected. These requirements were confirmed by a second series of single-deletion experiments in which only the "required" vitamins were tested. After incubation for 1 week, serial transfers were made from and into the same deficient medium to eliminate the possibility of vitamin carry-over. The growth yield was then determined and compared to the appropriate control. This procedure was repeated for at least three successive transfers before termination of the experiments. When no growth occurred in the absence of the vitamin, the requirement was considered absolute. A vitamin was considered stimulatory if, in its absence, the yield was less than that of the control.

The utilization of over 50 carbon sources (final concentration 0.02%, w/v, except where noted in Table 7) was tested by addng them to complex media. Strains 3a, 3b, and 9b were grown on the GY medium (excluding glucose) and strain 4a was grown on 0.01% YAmG (excluding glucose). Each carbon source was sterilized by autoclaving at 0.04% in water, prior to the addition to sterile medium. Ethyl alcohol and methanol were sterilized by membrane filtration. The sodium salts of acids were used, except for lactate (Ca salt). Cultures were incubated on shakers at 30 C for 1 week, and the yield was compared to a control with no added carbon source.

In testing for anaerobic growth, the procedure of Hungate (16) was used. Oxidative or fermentative utilization of glucose was determined by the Hugh-Leifson test (15).

The deoxyribonucleic acid (DNA) base composition, moles per cent of guanine plus cytosine (GC) of moles of total bases, was determined by M. Mandel with the buoyant density-CsCl method (28).

RESULTS

Direct observations of microorganisms in samples of freshwater. Freshwater samples were collected aseptically from the Putah Creek overflow in Davis, Calif. The concentrated samples (*see* Materials and Methods) were examined directly in the electron microscope. Objects the size of bacteria with several appendages extending from the surface were observed. These unusual forms were interpreted as microorganisms. The diversity of these forms is illustrated in Fig. 2–11.

The first electron micrograph (Fig. 2) shows a microorganism with many fine (diameter ca. 0.1 μ), slightly tapering appendages having transverse striations. A similar cell is shown in Fig. 3, but the appendages are fewer and longer (6 versus 4 μ) and no striations are detectable. In both organisms, the appendages lie in one plane and the cells are either spherical or disc-shaped. This will be referred to as form 1.

The cell shown in Fig. 4 appears to be lysed. Its general shape is similar to that of the organisms of form 1, but the appendages are only 2 μ long and extend in all directions from the cell. This will be referred to as form 2.

Figure 5 shows a form very frequently encountered in the direct examinations of the water samples. The principal distinguishing characteristic of this form is the marked tapering of the appendage from a diameter of over 0.25 μ to a blunt tip. The appendages extend in all directions from the cell to a length of 1 to 2 μ. This will be referred to as form 3.

Figures 6, 7, and 8 show organisms that are designated as form 4. The appendages of this

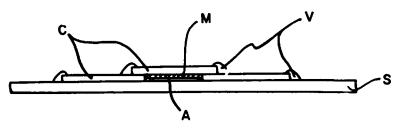
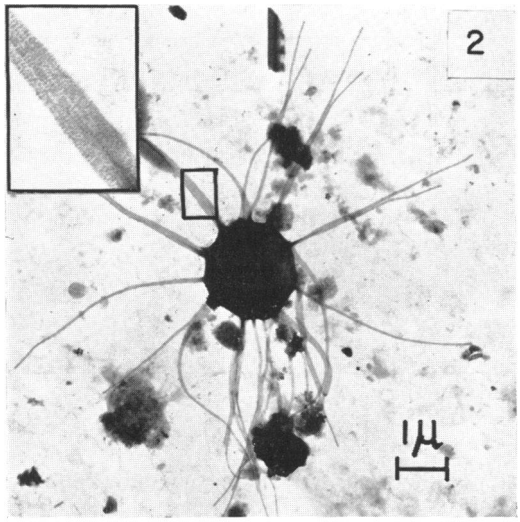


FIG. 1. *A cross section of the slide culture preparation showing the arrangement of components. Medium, M; vaspar, V; coverslip, C; airspace, A; slide, S.*



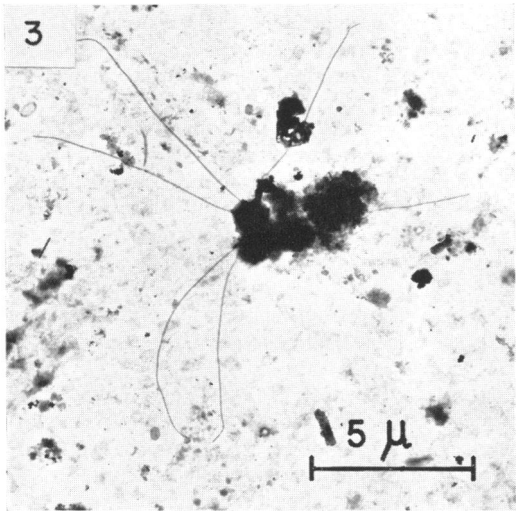


FIG. 2. *Electron micrograph of a microorganism with many fine (diameter ca. 0.1 μ), slightly tapering appendages having transverse striations.*

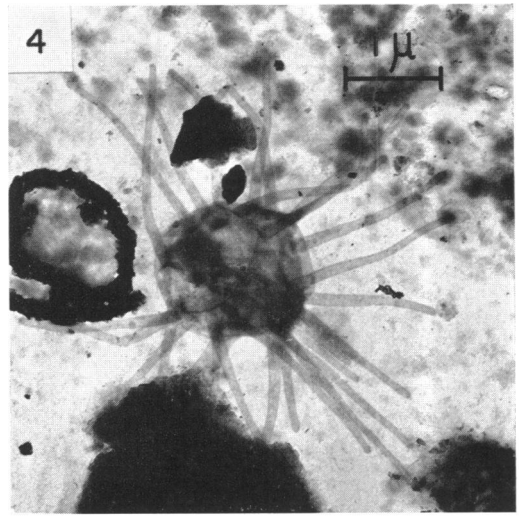
FIG. 3. *Similar cell to the one shown in Fig. 2, but the appendages are fewer and longer (6 versus 4 μ) and no striations are detectable.*

form are about 3 μ long; their diameter, about 0.2 μ, is slightly wider at the basal portion. In Fig. 6, cytoplasm can be seen to extend partway into the appendages of the cell. Many specimens of the organism shown in Fig. 7 have been observed in the phase contrast microscope. This cell has four appendages that extend in all directions; the longest appendage bears a bud (Fig. 7). Note that in the left half of the cell shown in Fig. 8 there is a small extension of cytoplasm for each appendage.

The appendages on the cell shown in Fig. 9 are much more electron transparent than those of the other cells. The cell itself is definitely rod-shaped. This will be referred to as form 5.

The cells shown in Fig. 10 have very fine appendages of various lengths, possibly pili, radiating from the spherical cells. This will be referred to as form 6.

The rod-shaped cell shown in Fig. 11 has very rigid appendages, some of which appear to be broken. This will be called form 7.



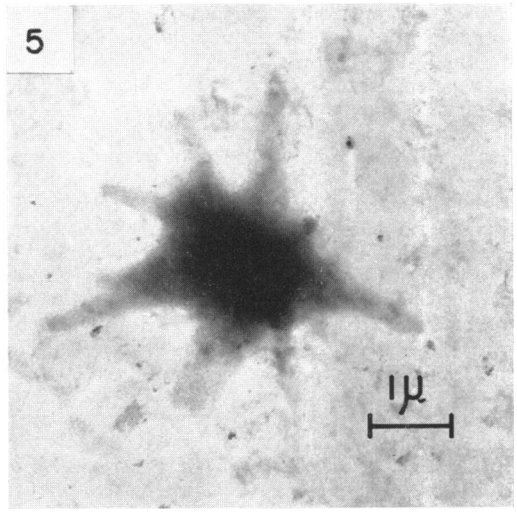
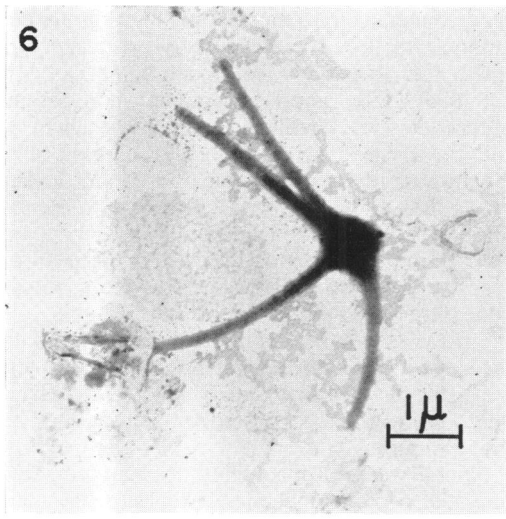


FIG. 4. *Cell appears to be lysed; its general shape is similar to that of the organisms shown in Fig. 2 and 3, but the appendages are only 2 μ long and extend in all directions from the cell.*

FIG. 5. *Principal distinguishing characteristic of this form is the marked tapering of the appendages from a diameter of over 0.25 μ to a blunt tip.*



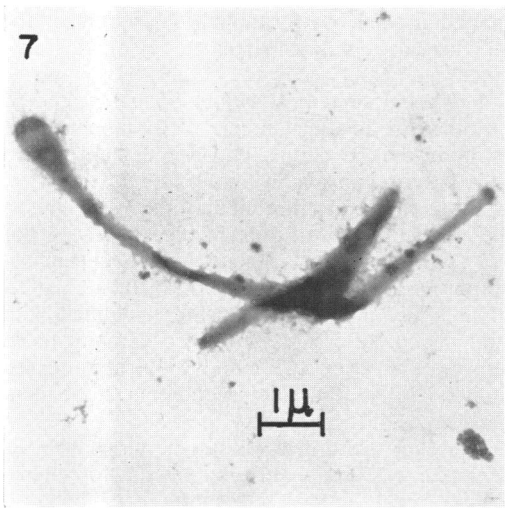


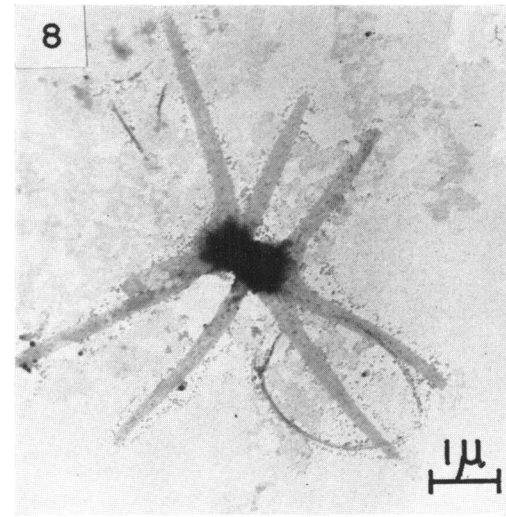
FIG. 6. *Cytoplasm can be seen to extend part way into the appendages of the cell.*

FIG. 7. *This cell has four appendages that extend in all directions.*

These electron micrographs indicate the existence in water of a variety of hitherto undescribed microorganisms having remarkable cell shapes. Other forms (8 and 9) appeared later during the course of isolation attempts.

Cultivation of some organisms with multiple appendages. Freshwater samples were obtained from the creek in Davis and a slough near Dixon, Calif. Peptone was added to each sample to a final concentration of 0.01%, according to Houwink's enrichment procedure for *Caulobacter* (13). The samples were incubated at room temperature and were examined periodically with a Zeiss GFL phase-contrast microscope.

Objects of bacterial size with appendages radiating from their surfaces were first seen after about 2 weeks of incubation. One of these forms was frequently encountered in both the peptone-creek (PC) and the peptone-slough (PS) cultures. Subsequently, this form increased in number. Numerous 1 μ long "spines" extend from the surface of this form, giving it the appearance of a microscopic burr (Fig. 12). The length and taper



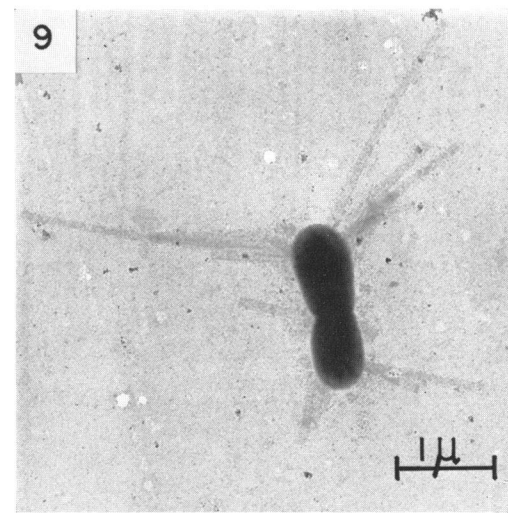
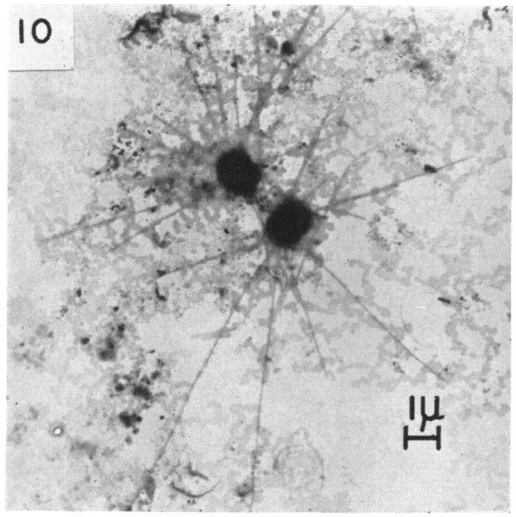


FIG. 8. *In the left half of the cell, there is a small extension of cytoplasm for each appendage.*

FIG. 9. *Appendages of this cell are much more electron transparent than those of the other cells. The cell itself is rod-shaped.*



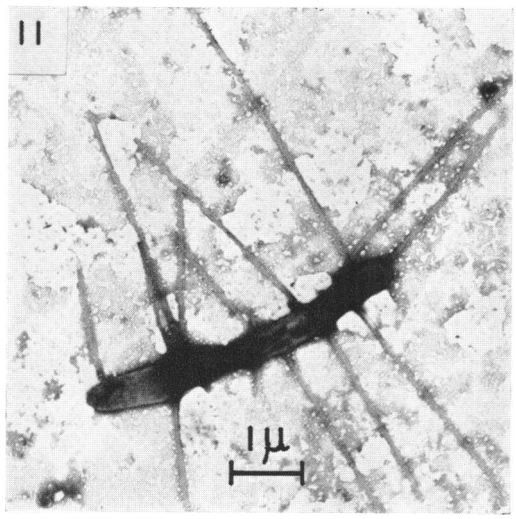


FIG. 10. *Cells have very fine appendages of various lengths, possibly pili, radiating from the spherical cells.*

FIG. 11. *This rod-shaped cell has very rigid appendages, some of which appear to be broken.*

of the appendages are suggestive of form 3 (Fig. 5).

A second form was seen in the PC culture only a few times in these initial enrichment experiments. This organism has fewer but longer appendages (about 3 μ) than those of form 3. The phase photomicrograph (Fig. 13) indicates that this is a representative of form 4 (Fig. 6, 7, and 8).

Another form, which was different from any seen in the direct observations was discovered. This form (Fig. 14) has six appendages lying in one plane and resembles a six-pointed star. This type, termed form 8, was first observed in the PC culture after 3 weeks of incubation. A week later, it was also observed in the PS culture.

Subsequently, another series of enrichment experiments was conducted. Water samples from the Davis creek were aseptically collected, and 100 ml was used to inoculate (i) a 250-ml Erlenmeyer flask autoclaved with 10 mg of dry peptone and (ii) a 250-ml Erlenmeyer flask autoclaved with 10 mg of dry yeast extract and 10 mg of L-fucose and to which 100 μg of streptomycin was added after the flask had cooled.

Similarly, two flasks (iii) and (iv) containing the same ingredients, respectively, as (i) and (ii), were autoclaved with 100 ml of MS + DW. Here, only 1 ml of the creek water sample was used as the inoculum.

After 3 weeks of incubation at room temperature, the peptone-mineral salts culture (iii) contained many organisms of forms 3 and 4. Star-shaped organisms (form 8) were found in the YE-fucose-streptomycin culture (ii) at the same time.

Isolation of organisms with multiple appendages. The increase in the numbers of the appendaged forms in the PC culture encouraged attempts to isolate the organisms in pure culture. The initial attempts were concentrated on form 3 since it was the most abundant of the new types. Dilutions of PC (100-fold) were sprayed, with a chromatograph spraying apparatus, onto 0.05% PCA (0.05% peptone in creek water solidified with 1.5% agar). After 1 week of incubation at room temperature, over 100 colonies were examined individually in the microscope, but none contained cells of form 1.

The slide culture technique developed by R. Martucci was used to determine which of several media might support the growth of form 3. Microcolonies developed only on 0.01% PCA, indicating that it could be useful in subsequent attempts at isolation.

Petri plates of 0.01% PCA were sprayed with 100-fold dilutions of the culture. After 4 days of incubation at room temperature, the colonies visible to the naked eye were marked. It was assumed that the slow-growing appendaged organisms would not appear within this time. The plates were incubated for another week, and then each of the new (i.e., unmarked) colonies was examined microscopically. Two white colonies contained cells similar to form 3, but their cell surface projections were not as long. Organisms having this morphology will be referred to as form 9. One of these colonies, strain 9a (Fig. 15), was restreaked until pure. It did not grow well on any medium tested.

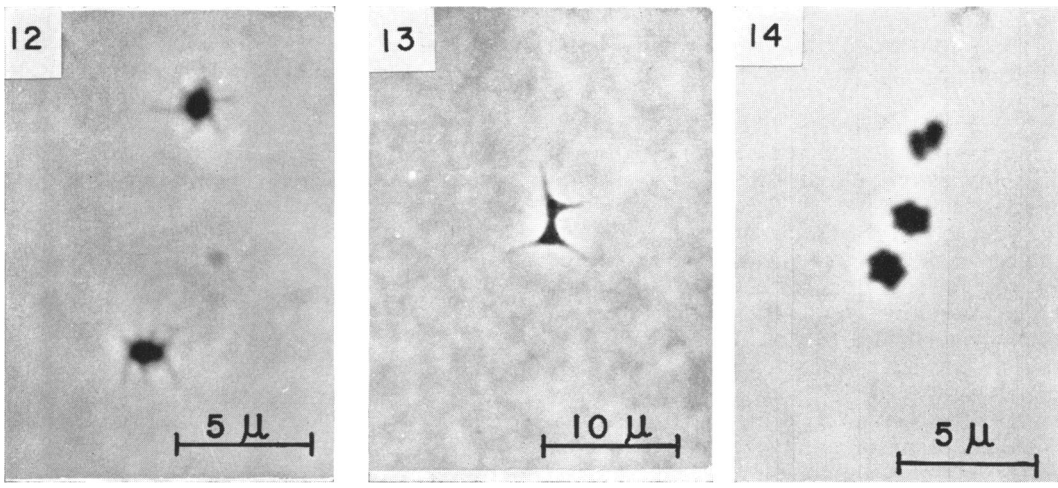


FIG. 12. *Phase photomicrograph of form 3 from a crude culture. Several 1 μ long appendages extend in al directions from the cell.*

FIG. 13. *Phase photomicrograph of form 4 with appendages approximately 3 μ long.*

FIG. 14. *Phase photomicrograph of two star-shaped cells (form 8) observed in a crude culture. Each has six appendages extending in one plane.*

Another yellow-pigmented organism (strain 9b) was found on a 0.01% PCA plate which had been sprayed with a dilute suspension of scrapings from a slide submerged in creek water. It grew well and has been further characterized. A seemingly identical strain was isolated 2 years later from the same creek, but it has not been maintained.

A red-pigmented isolate of form 9, strain 9c, was also isolated from the creek. Although it grew well initially, subcultures onto the same medium failed to grow.

An interesting observation was made while examining wet mounts of the second series of enrichment cultures that had been incubated for 30 days. Although most of the nonappendaged cells in culture (iii) were attached to the surface of the coverslip or the slide, the forms with multiple appendages drifted with the current. This suggested that the appendaged forms might be separated from the attaching organisms by exposing the suspension to an extensive glass surface. Thus, a chromatographic column containing glass beads was employed, and 1 ml of culture (iii) was passed through it. Alternate drops eluted from the column were used to inoculate liquid cultures and to spread on plates. After 3 weeks of incubation, cultures inoculated with the first two drops were sterile. The liquid culture inoculated with the third drop contained many appendaged forms, and three white colonies containing appendaged cells were found on the plate spread with the fourth drop. Two of the colonies contained organisms of form 3. Most of the cells from one of these colonies, now termed strain 3a, contained refractile areas; cells from the other colony (strain 3b) did not. The third colony contained organisms having the 3-μ appendages characteristic of form 4. It will be referred to as strain 4a.

The glass bead procedure was unsuccessfully applied to the isolation of the star-shaped organism from culture (ii). But, for morphological

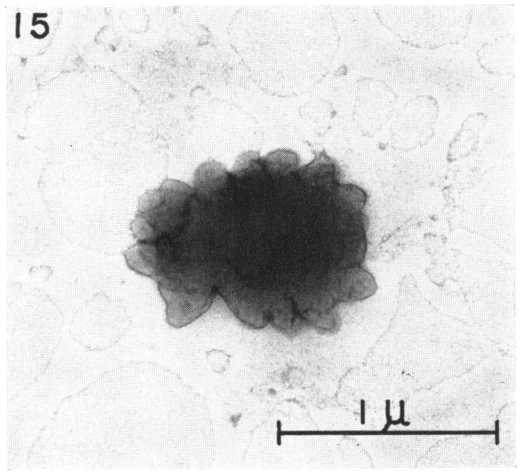


FIG. 15. *Electron micrograph of a PTA-stained cell of strain 9a.*

investigation, a droplet of this culture was placed on a Formvar-coated grid, stained with PTA, and observed directly in the electron microscope (Fig. 16).

It seemed reasonable that the glass-bead technique might be successfully used to isolate such organisms directly from their natural environment; this procedure would thus eliminate the need for lengthy incubations and would also provide some indication of the numbers in the habitat. The cells from creek water were concentrated 60-fold by centrifugation. This suspension (0.2 ml) was added to a sterilized column containing glass beads and was treated as described previously. No forms with appendages were, however, isolated.

Table 2 summarizes some of the information on these forms with multiple appendages.

As a preliminary to more detailed studies of morphology, several culture ingredients were tested. Yeast extract, added to MS, gave better growth than peptone, and growth was improved still further by the addition of glucose. A medium (0.01% GY) containing 0.01% glucose, 0.1% yeast extract, and MS supported good growth of strains 3a, 3b, and 9b. Strain 4a grew well on YAmG (0.01 to 0.025% yeast extract, 0.025% ammonium sulfate, 0.025% glucose, and MS).

Characteristics of isolated strains. Some major properties are common to all of the strains isolated. All are procaryotic and gram-negative. Thin sections reveal a trilaminate cell wall of the type found in many other gram-negative bacteria. The appendages characteristic of these strains are part of the cell, bounded by the cell wall. Each form is described individually; the important differential characteristics are summarized in Table 3.

Form 3. When organisms of this form were observed in enrichment cultures, very few contained refractile vacuoles. As mentioned previously, strain 3a did have vacuoles when first isolated, although strain 3b did not. Figures 17 and 18 are electron micrographs of whole cells from the initial isolated colonies of 3a and 3b, respectively. After continued cultivation, a large proportion of cells of both strains have always been observed to contain these vacuoles, and the vacuolated area within the cells was increased in size (Fig. 19 and 20). When observed in the electron microscope,

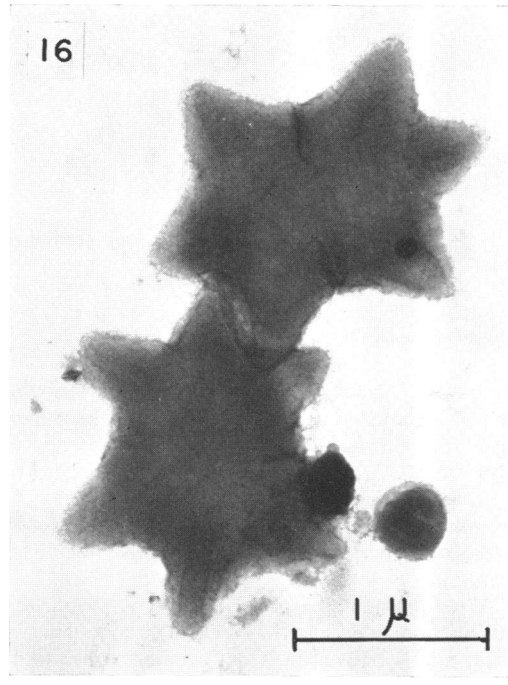


FIG. 16. *Electron micrograph of star-shaped cells (form 8) observed in a negatively stained preparation from a crude culture. Note the division planes.*

TABLE 2. *Forms with multiple appendages observed in water samples and in crude cultures*

Form	Figure	Cell shape[a]	Length of appendages	Positive cultures	Strains isolated	Strains lost
			μ			
1	2, 3	Sphere or disc	<4			
2	4	Sphere	ca. 1			
3	5, 12	Sphere or short rod	<2, tapers	PC, PS, iii, iv	3a, 3b	
4	6, 7, 8, 13	Sphere, etc.	ca. 3	PC, iii	4a	
5	9	Rod	ca. 3			
6	10	Sphere	variable			
7	11	Rod	ca. 4			
8	14, 16	Star	ca. 1	PC, PS, ii		
9	15	Rod	<0.5	PS	9a, 9b, 9c	9a, 9c

[a] The shape of the cell excluding the appendages (except in form 8).

TABLE 3. *Characteristics of isolated strains*

Characteristic	Strains		
	3a and 3b	4a	9b
Gram stain	Negative	Negative	Negative
Maximal appendage length/cell diameter	Approx 1.0[a]	Approx 3.0	0.5
Mode of division	Binary fission	Budding	Binary fission
Gas vacuoles	Present	Present	Absent
Motility	Absent	Absent	Present
Oxygen relations	Obligate aerobe	Euryoxic	Obligate aerobe
GC ratio	69.4, 69.9		65.8

[a] Occasionally, an abnormally long appendage is found.

the vacuole is resolved into subunits or vesicles that have a characteristic size and morphology; each subunit (ca. 100 × 300 mμ) consists of a short cylindrical section with a cone at each end. The phase photomicrograph (Fig. 21) shows the appearance of cells containing vacuoles in the light microscope: the subunits are too small to be individually resolved. The low refractive index of the vacuoles can no doubt be explained by the fact that these structures contain gas. It was first thought that these structures were lipid inclusions, but their fine structure is homologous with that of the gas vacuoles of *Halobacterium* (14, 32), the blue-green algae (5), and the photosynthetic bacteria (23).

The diameter of the cell, exclusive of appendages, is about 1.0 μ. Several conical appendages radiate from the surface, each approximately 0.75 μ long, and with a basal diameter of 0.25 μ, tapering outward to a blunt tip. Occasionally, a cell has a much longer appendage (ca. 2.5 μ), such as the one shown in Fig. 22. Extended laboratory cultivation has been accompanied by a decrease in the number of appendages per cell; some cells now have none. The organism is nonmotile.

Thin sections of strain 3a (Fig. 23) and 3b indicate that these organisms are procaryotic. What appear to be short segments of unit membrane are found throughout the cytoplasm; the enlarged area in Fig. 23 is an example. These structures are interpreted not as unit membranes, but as the membranes bounding gas vacuoles that have collapsed during preparation of the specimen (5). Cytoplasm extends into some of the appendages.

Colonies on plates streaked with this organism (Fig. 24) vary from large, translucent and white, to small, opaque and chalky-white. The variability is due, at least in part, to differences in the number of gas vacuoles within the cells of the colony. Vacuolated cells are more abundant in the opaque, chalky-white colonies (8). All colonies have a circular form, an entire margin, and their elevation is raised to convex.

Cells grown in a liquid medium on a shaker are evenly suspended. In stationary cultures, however, cells accumulate to form a loose surface

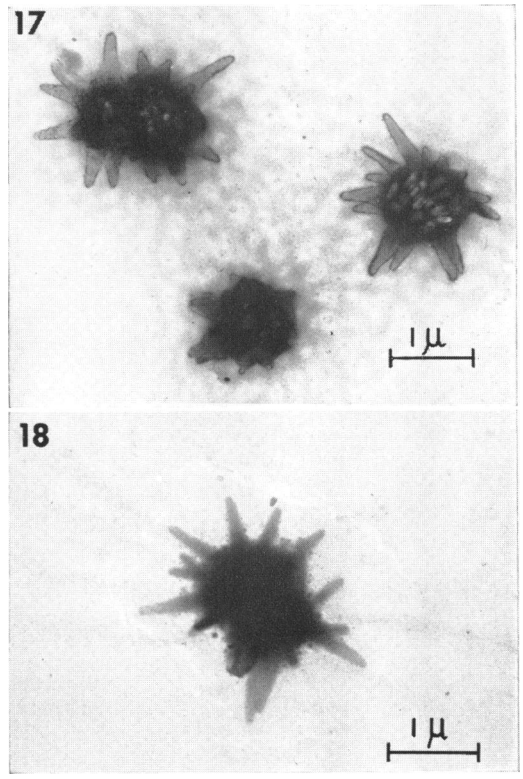
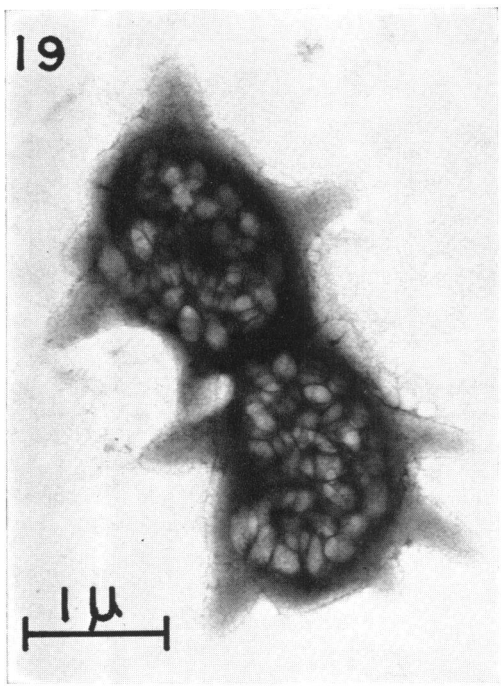
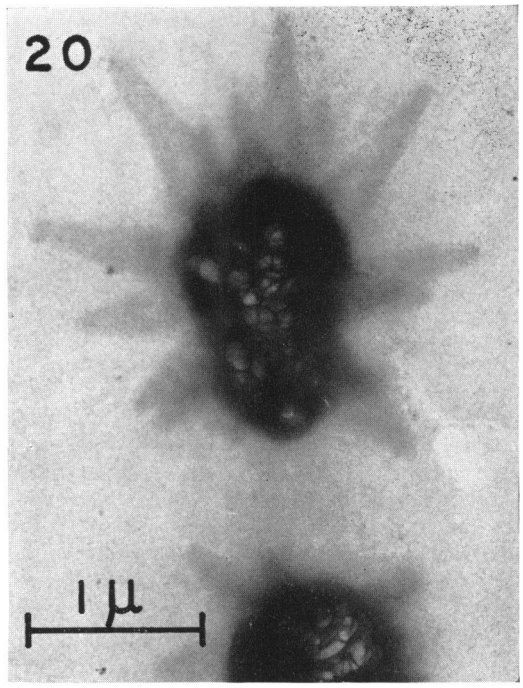


FIG. 17. *Electron micrograph of negativey stained cells of strain 3a from the original isolation colony. Note the electron-transparent inclusions, interpreted as the vesicles of gas vacuoles.*

FIG. 18. *Electron micrograph of a negatively stained cell of strain 3b from its original isolation colony. Note the absence of gas vacuoles.*





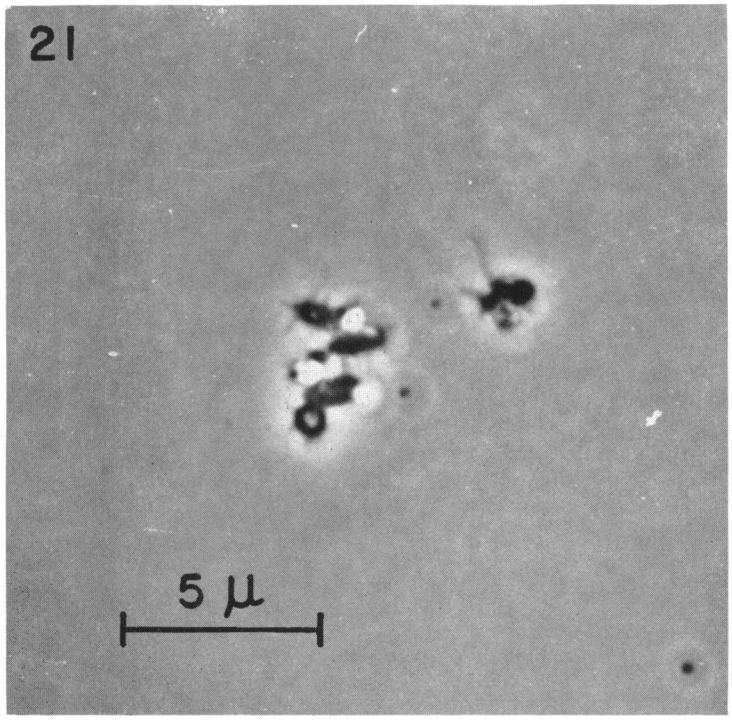


FIG. 19 and 20. *Electron micrographs of negatively stained cells of strains 3a and 3b. Note the increase in vacuolar area in 3a and the presence of vacuoles in 3b.*

FIG. 21. *Phase photomicrograph of strain 3a showing the appearance of the gas vacuoles as refractile areas in the cells. The subunits are not individually resolved in light microscopy.*

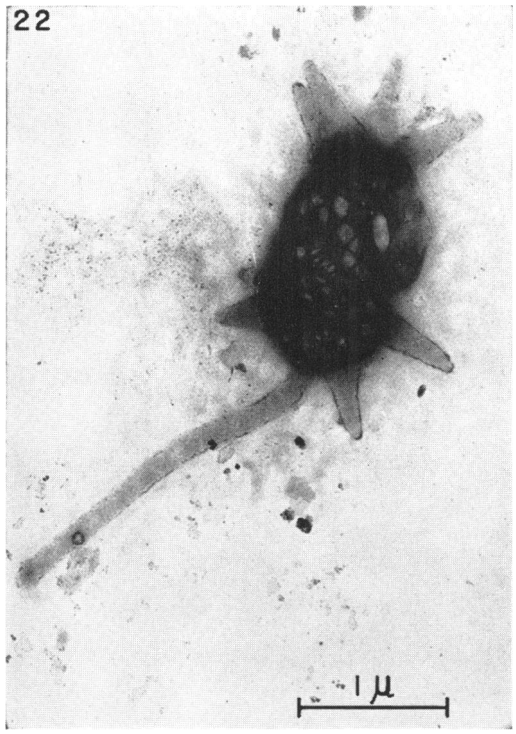


FIG. 22. *Electron micrograph of a cell of strain 3a that has an unusually long appendage (about 2.5 μ). These long appendages, less frequent than the shorter ones, do not taper toward the tip.*

layer, presumably because the gas vacuoles make them buoyant.

The minimal growth temperature for strains 3a and 3b was 9 C; the maximum was between 42 and 45 C. Good growth occurred between *p*H 6.0 and 7.2 on the YE medium buffered with 0.04 M phosphate. The greatest yields were obtained at *p*H 6.0 and 6.5; lower *p*H values have not been tested. Neither strain could grow under strictly anaerobic conditions in a medium composed of 0.1% Casamino Acids, 0.02% glucose, MS, and vitamins, with or without potassium nitrate. Glucose was utilized oxidatively (not fermented).

The DNA of strains 3a and 3b contains, respectively, 69.4 and 69.9 moles per cent of GC.

Form 4. The most obvious distinguishing characteristic of form 4 is the length (3 μ) of its appendages, approximately three times the diameter of the cell. Figures 25, 26, and 27 are phase and electron micrographs of the single strain, 4a, representing this group. The diameter of the appendages is about 0.25 μ, newly forming "arms" being slightly thicker. There may be from two to eight appendages per cell. Annular rings or "Querbalken" have never been observed. Occasionally, an appendage is bifurcated (Fig. 28). The tips distal to the bifurcation are not always the same length. The position of the fork along the appendage is variable.

Electron micrographs indicate that this organism sometimes has gas vacuoles in its cytoplasm (Fig. 29). Like those in strains 3a and 3b, they are composed of vesicles, similar to the structures reported for other procaryotic organisms with gas vacuoles (5, 14, 23). The proportion of cells containing gas vacuoles is much smaller than in the strains of group 3 and depends somewhat on the conditions of cultivation. Note that the subunits may extend some distance into the appendages (Fig. 29). Strain 4a is nonmotile.

Thin sections (Fig. 30) reveal that this organism is procaryotic. Ribosomes and other cellular material are contained within the cell wall and membrane of the appendages.

The development of the appendages and their distribution to the daughter cells were followed in a slide culture. Figures 31 and 32 show the growth of two cells over a period of several hours. The sequential photomicrographs reveal that the cell divides by budding, a major distinctive character of this group. The photomicrographs provide evidence for the following steps during division: (i) extension of a bud from the mother cell; (ii) differentiation of the bud into a cell body with two or more arms, accompanied by an increase in its size; and (iii) separation of the cells after the bud has attained the approximate size and shape of the mother cell.

Figure 33 is a diagram of the division process. A bud always develops from the same position on the mother cell. As in *Caulobacter*, the mother cell can be identified by the presence of the old appendages, which remain with it; the daughter cell never acquires an appendage directly from the mother cell. As in other budding organisms, the cell wall of the bud is newly synthesized.

Strain 4a grew at a temperature of 39 C but not at 42 C. The minimal growth temperature was between 6 and 9 C. The optimal *p*H for growth was between 6.9 and 7.3, as determined with the YE medium. Growth occurred between *p*H 6.3 and 7.5; a wider range was not tested.

Strain 4a is weakly catalase positive. Unlike the isolates of groups 3 and 9, strain 4a is euryoxic (facultatively anaerobic) and can grow under strictly anaerobic conditions in a medium composed of 0.025% ammonium sulfate, 0.025% glucose, 0.025% Casamino Acids, MS, and vitamins. Potassium nitrate did not improve the anaerobic growth. The organism can utilize glucose fermentatively.

Form 9. The surface of this form has a jagged or sawtooth appearance, as observed in the phase microscope. Because of the small size of the

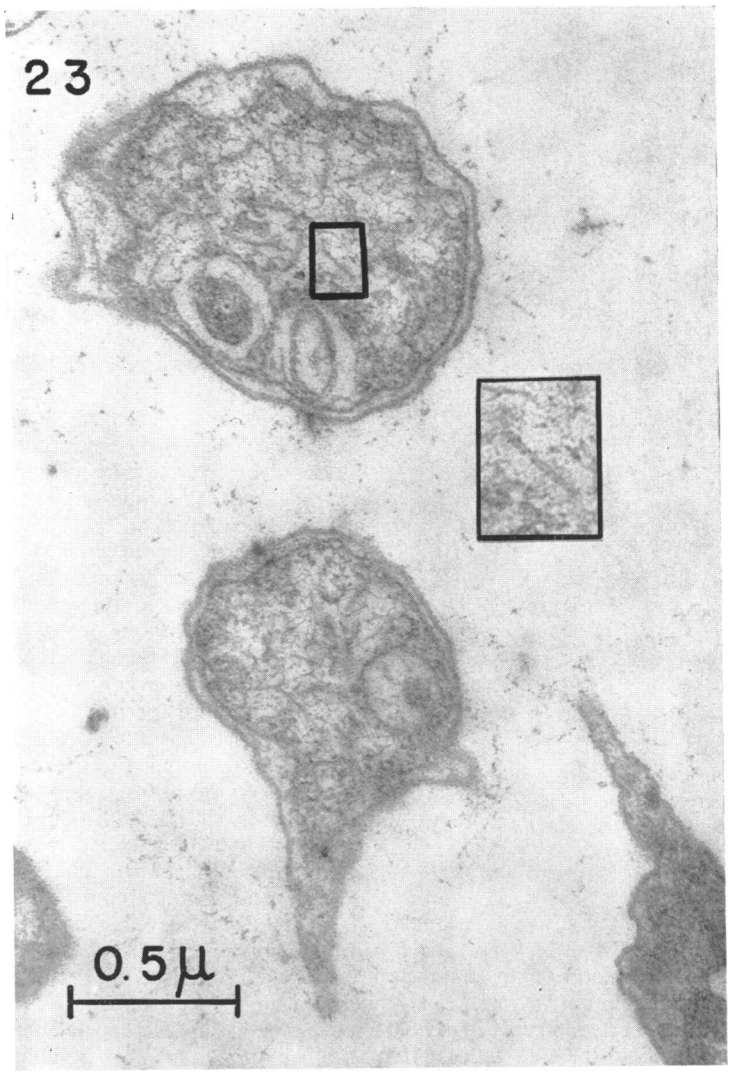


FIG. 23. *A thin section of strain 3a. The "unit membrane" of the enlarged area is interpreted as a collapsed subunit of the gas vacuole.*

appendages, they are poorly defined (Fig. 34). Electron micrographs (Fig. 35) of whole cells of strain 9b (as well as strain 9a, *see* Fig. 15) revealed that the extensions are rounded, giving the surface a lumpy appearance. The appendages are approximately 0.25 μ in diameter and somewhat longer than 0.25 μ, extending in all directions from the cell. The length of the cell (exclusive of appendages) is about 1.5 μ and its diameter is about 1.0 μ).

This strain is motile and possesses a single polar to subpolar flagellum, as shown in the electron micrograph (Fig. 35). The motility is peculiar, cells tumble over themselves (somersault) or travel about in circles. The red-pigmented strain, 9c, exhibited the same phenomenon. Perhaps the surface irregularities are responsible for this unusual motility, or it may be due to the displacement of the flagellum from a polar position.

No inclusions or vacuoles have been observed in cells of this group.

Thin sections (Fig. 36) of strain 9b indicate that the organism is procaryotic. Ribosomes and nuclear strands can be seen throughout the cytoplasm which occasionally extends into an appendage.

Colonies of strain 9b have a yellow pigment.

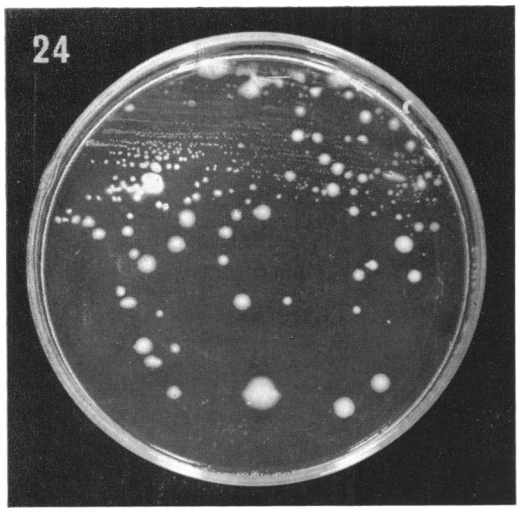


FIG. 24. *Culture of strain 3a showing its colonial variation.*

Colonies of strain 9c, the only other pigmented isolate, were red.

On 0.05% GY medium, the colonies of 9b have a circular form, convex elevation, and an entire margin. In stationary liquid medium, growth is initially evenly suspended, then cells settle to the bottom of the flask; no cells accumulate at the surface. In the 0.05% GY medium, the generation time at 30 C was 6.3 hr.

The maximal growth temperature is about 37 C, and the minimum is about 9 C. The *p*H optimum for this strain is about 7.0. This was determined on the YE medium. There was no growth below *p*H 6.6. The alkaline limit was not determined. Strain 9b did not grow under strictly anaerobic conditions either in the presence or absence of glucose or potassium nitrate. It is catalase positive and metabolizes glucose oxidatively.

The DNA composition of strain 9b is 65.8.

Nutrition. None of the isolated strains grew in a medium free of vitamins. Strains 3a and 3b were carried through three transfers on a Vitamin Free Casamino Acids-glucose medium (0.025% GCA) supplemented with biotin, thiamine, B_{12}, and folic acid; folic acid was stimulatory (perhaps essential in strain 3a), whereas the other three components were essential. Strain 4a was grown on a similar vitamin-free medium (GCAAm), containing five vitamins, through four successive transfers. Pantothenic acid was absolutely required for growth; biotin, folic acid, thiamine, and nicotinamide stimulated growth. Strain 9b was carried through three successive transfers on the vitamin-free 0.05% GCA medium supplemented with biotin, thiamine, and pantothenate. This strain has an absolute requirement for thiamine, and its growth is stimulated by biotin.

Growth yields on several media are shown in Table 4. In contrast, with yeast extract, supplementation of nutrient broth with glucose did not increase the yield of strains 3a, 3b, or 4a (these results are not included in the table).

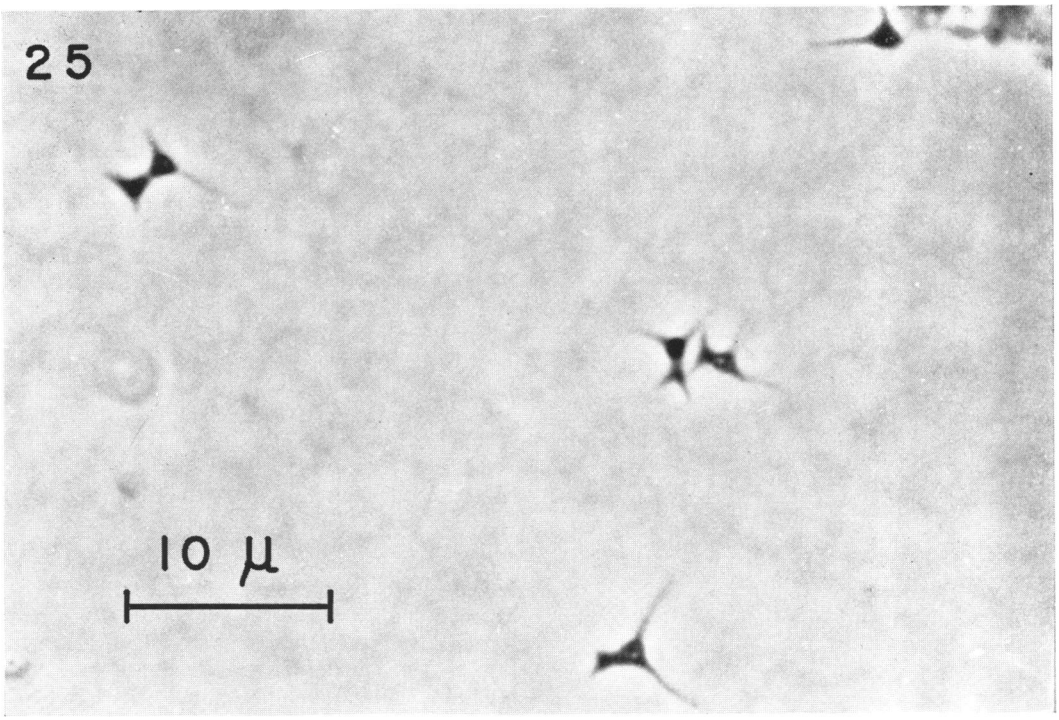
Casamino Acids, Casitone, and casein were tested as the sole source of nitrogen (Table 5). None of the organisms could utilize casein as the sole substrate, but if glucose was added, all three organisms grew to turbidities comparable to those with 0.1% Casamino Acids. All of the organisms used ammonium sulfate, but not potassium nitrate, as a sole source of nitrogen (Table 6).

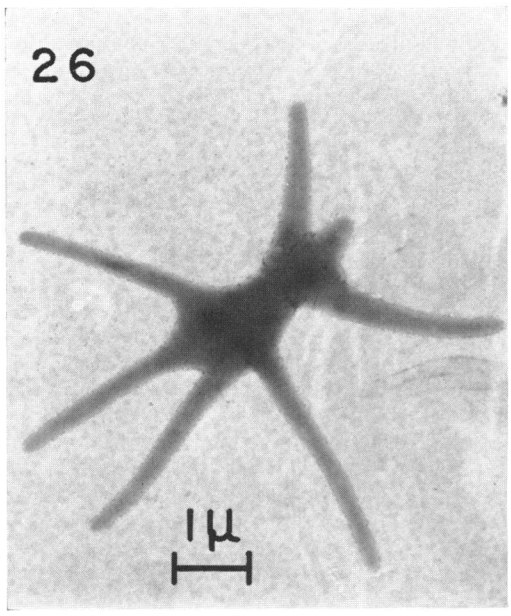
Table 7 lists the carbon sources that were tested and their utilization by the isolates.

DISCUSSION

The results of this study indicate that freshwater contains previously unknown microbial forms. Apparently, these microbial forms are not restricted to aquatic habitats since Stefanov and Nikitin (31) and Nikitin et al. (21) have encountered similar forms when examining aqueous extracts of soil with the electron microscope. The first paper (31) contains an electron micrograph of a "submicroscopic body" resembling strain 4a, except that the appendages have transverse striations. The second paper (21) contains illustrations of forms resembling strains 3a and 3b and the star-shaped organism, form 8. The authors were not able to cultivate these microorganisms.

Are these microorganisms procaryotic? Nikitin et al. (21) thought that the organisms they observed (that resemble form 3) were small, amoeboid protozoa (one measures 1.0 × 0.7 μ), and assumed that the surface extensions were pseudopodia. They postulated that the star-shaped organism was also a small protozoan, which developed into another larger form with tooth-like appendages distributed over the surface of the cell. It is true that these soil forms have shapes suggestive of protozoa, but a decision on their protozoan or bacterial affinities should be based on their fine structure (30). The electron micrographs of thin sections of our isolates (Fig. 23, 30, 36) show unmistakably that these organisms are procaryotic, and thus belong to the bacteria. It is highly probable that the alleged "protozoa" of Nikitin et al. (21), which resemble the forms we have seen, are also bacteria. If so, there must be numerous types of bacteria possessing multiple appendages, in soil as well as in water.





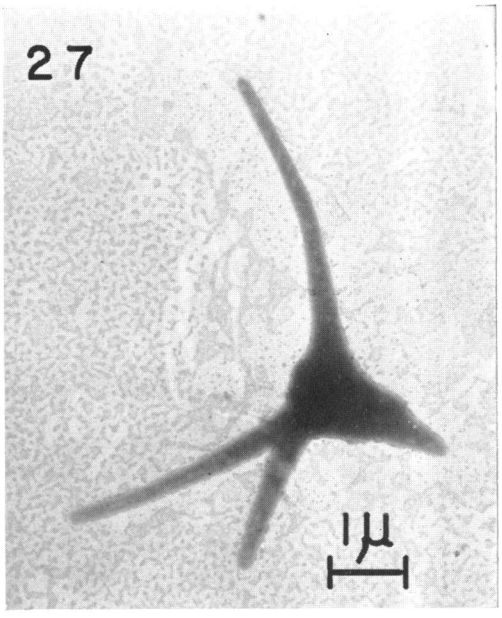


FIG. 25. *Phase photomicrograph showing the lateral view of several cells of strain 4a.*

FIG. 26 and 27. *Electron micrographs of negatively stained strain 4a. Figure 26 shows a cell prior to division, whereas Fig. 27 shows a cell after division.*

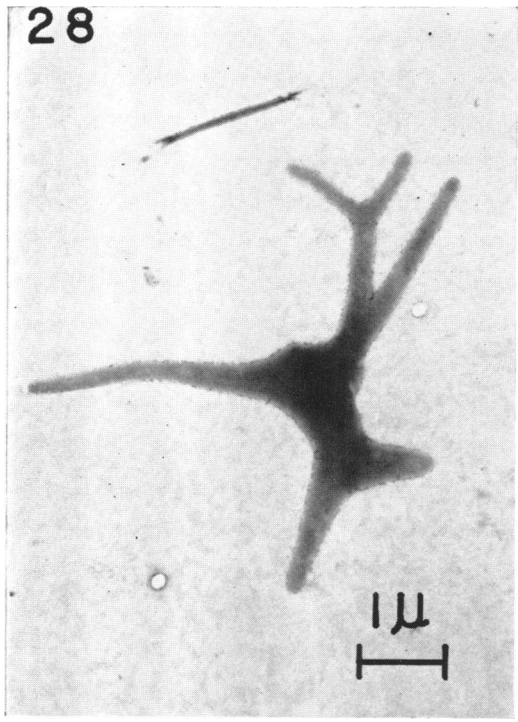


FIG. 28. *Electron micrograph of strain 4a showing a bifurcated appendage.*

If numerous multiple-appendaged bacteria occur in soil and water, why have they been discovered only recently? Are they rare, comprising only a minor fraction of the total bacterial flora? An occasional observation of such a form would not be sufficient to convince the viewer that he was seeing more than some anomaly. These bacteria could have been interpreted as involution forms of other bacteria, spores, pieces of debris, or even parts of protozoa. Henrici (11) may have overlooked them because he was examining the bacteria attached to surfaces rather than those in the open water. Or, as Stolp and Starr (33) suggest, discussing *Bdellovibrio*, "There was no *a priori* reason to expect the existence of this unusual creature."

Were these bacteria missed because they are not widely distributed? We have found these forms in peptone cultures of water from Beaver Creek, Priest Lake, Idaho, the ice-covered James River upstream from Huron, South Dakota, and Lake Lansing, Michigan. As yet, the distribution has not been systematically studied, but the fact that they have been found in soil on one continent and water in another indicates a wide, if not abundant, occurrence.

Definition of prostheca. The bacterial stalk was described by Henrici and Johnson (12) as a material "secreted from one side or one end" of the cell. Their order of stalked bacteria, Caulobacteriales (not presently recognized by *Bergey's Manual*), contained four families of stalked bacteria: *Nevskiaceae*, *Gallionellaceae*, *Caulobacteriaceae*, and *Pasteuriaceae*.

Houwink (13) showed that *Caulobacter* stalk, unlike that of *Gallionella*, *Nevskia*, and *Siderophacus*, is actually an extension of the cell, not a secretion from the cell. There are really, therefore, two kinds of stalks, the noncytoplasmic, secreted, extracellular stalk and the cellular stalk containing cytoplasm.

One other genus characterized by a "cellular stalk" has been isolated and described by Poindexter (24). The cells of this genus, *Asticcacaulis*, have a stalk in a subpolar position. The holdfast is produced at the pole of the cell as in *Caulobacter*. Thus, the stalk of *Asticcacaulis* is not involved in attachment, as it is in *Caulobacter*. For this reason, the stalk of *Asticcacaulis* has been termed a "pseudostalk" by Pate and Ordal (22). The fine structure of the "pseudostalk" is identical with that of the *Caulobacter* stalk (22, 25). Both consist of a core of membranous material, bounded by the cell membrane and the cell wall. The membranous core extends from a membranous organelle located at the junction of the cell and the stalk. Both the stalk and the pseudostalk have annular rings.

The only other procaryotic structure analogous to the "cellular stalk" of the caulobacters is the "hypha" or "filament" of *Hyphomicrobium* and

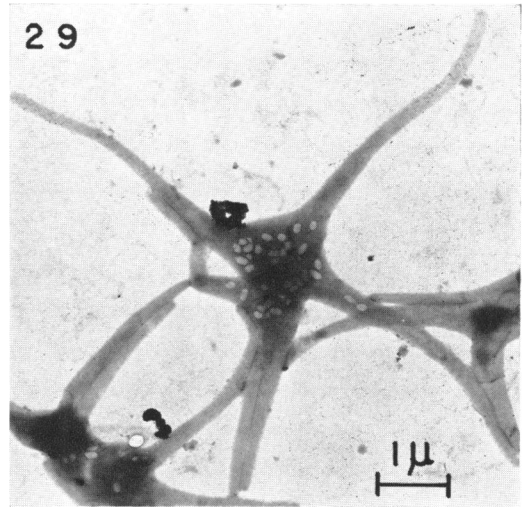


FIG. 29. *Electron micrograph of negatively stained cells of strain 4a containing gas vesicles.*

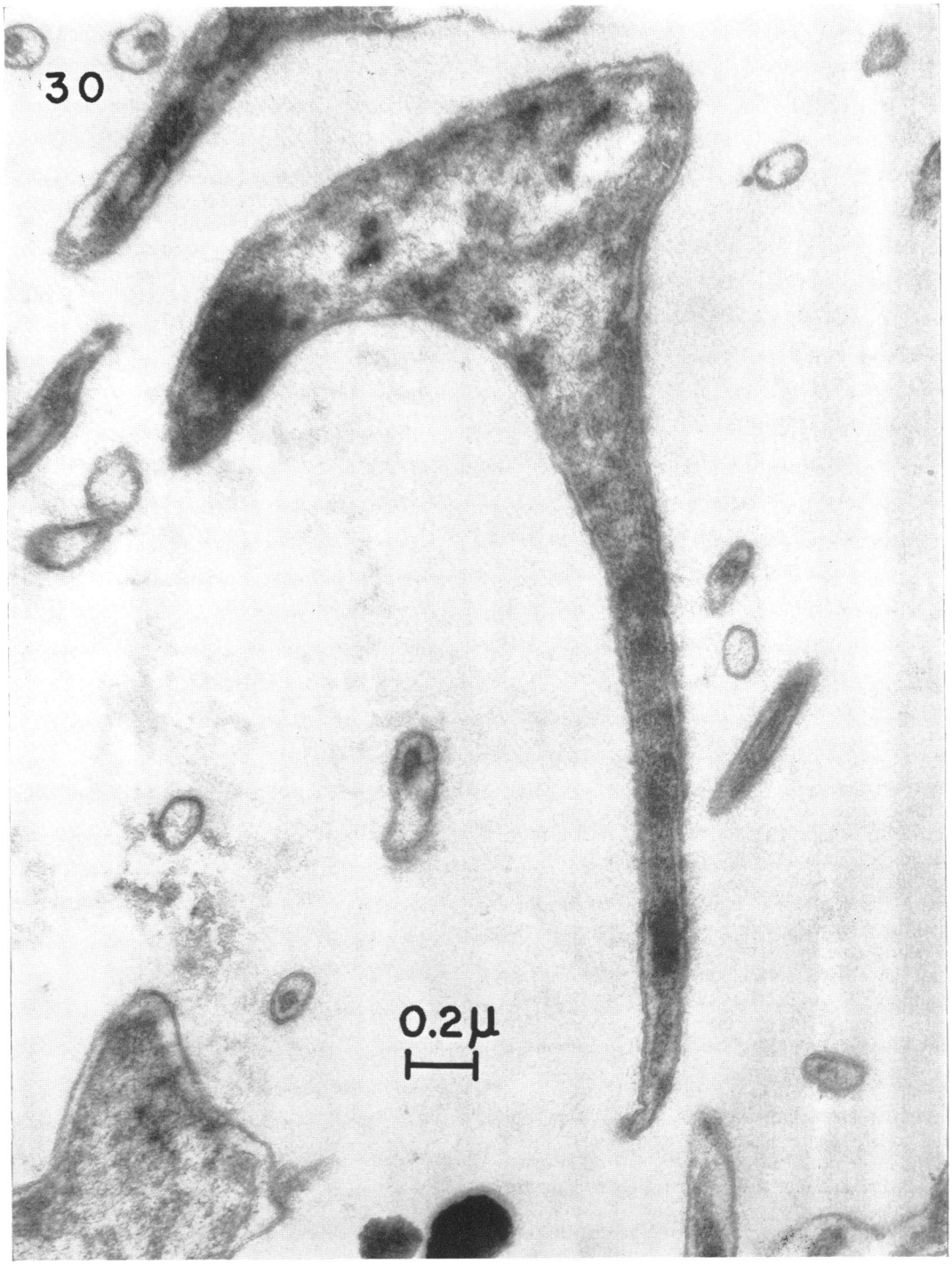


FIG. 30. *A thin section of strain 4a showing that it is a procaryotic cell with a cellular appendage.*

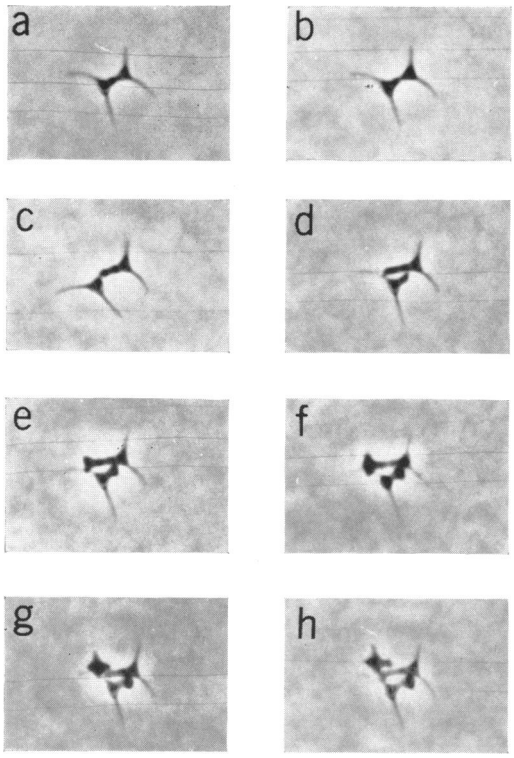


FIG. 31. *Sequential photomicrographs illustrating growth and division in the formation of a clone from a cell of strain 4a. The photomicrographs were taken at the following times (upper left to lower right): (a) 9:50 AM; (b) 11:57 AM; (c) 1:43 PM; (d) 3:19 PM; (e) 5:34 PM; (f) 6:46 PM; (g) 8:25 PM; (h) 10:58 PM.*

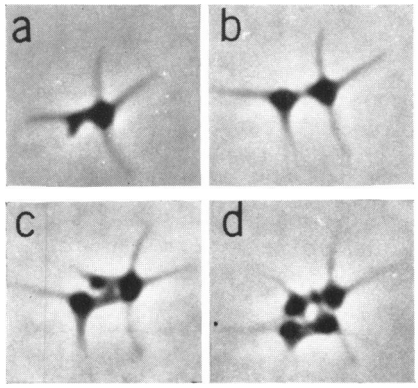


FIG. 32. *Sequential photomicrographs showing the development of a clone from a cell of strain 4a. The photomicrographs were taken at the following times: (a) 11:36 AM; (b) 1:35 PM; (c) 3:65 PM; (d) 5:03 PM.*

Rhodomicrobium. Like the stalk of the caulobacters, the hypha is a cellular appendage bounded by the cell wall. Unlike stalks, however, hyphae contain ribosomes and nucleic acid (4, 7). In addition, hyphae can bear buds and can

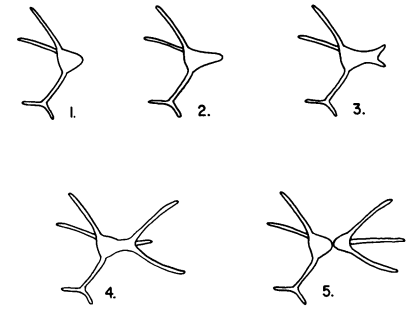
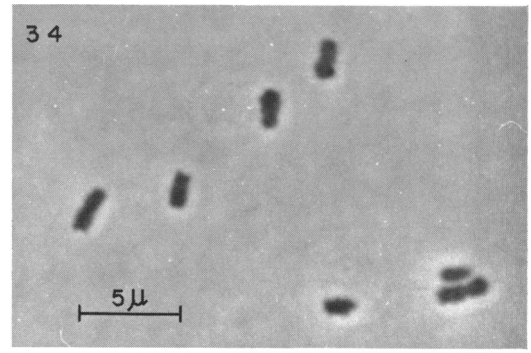


FIG. 33. *Sketch showing the division cycle in strain 4a. A cell (1) produces a bud (2) from which three arms differentiate (3 and 4). Division occurs when the mother and daughter cells are approximately the same size (5). One appendage is bifurcated.*



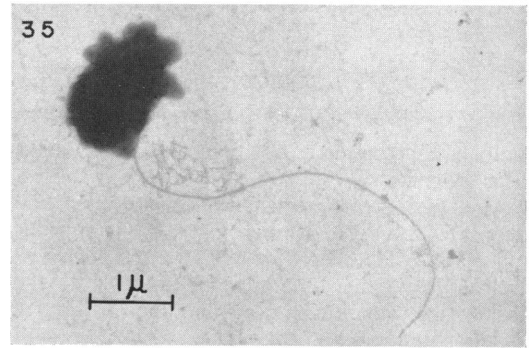


FIG. 34. *Phase photomicrograph of several cells of strain 9b. Note the uneven appearance of the surface.*

FIG. 35. *PTA-stained preparation of strain 9b. The surface irregularities are resolved and give the surface a "bumpy" appearance. There is a single flagellum.*

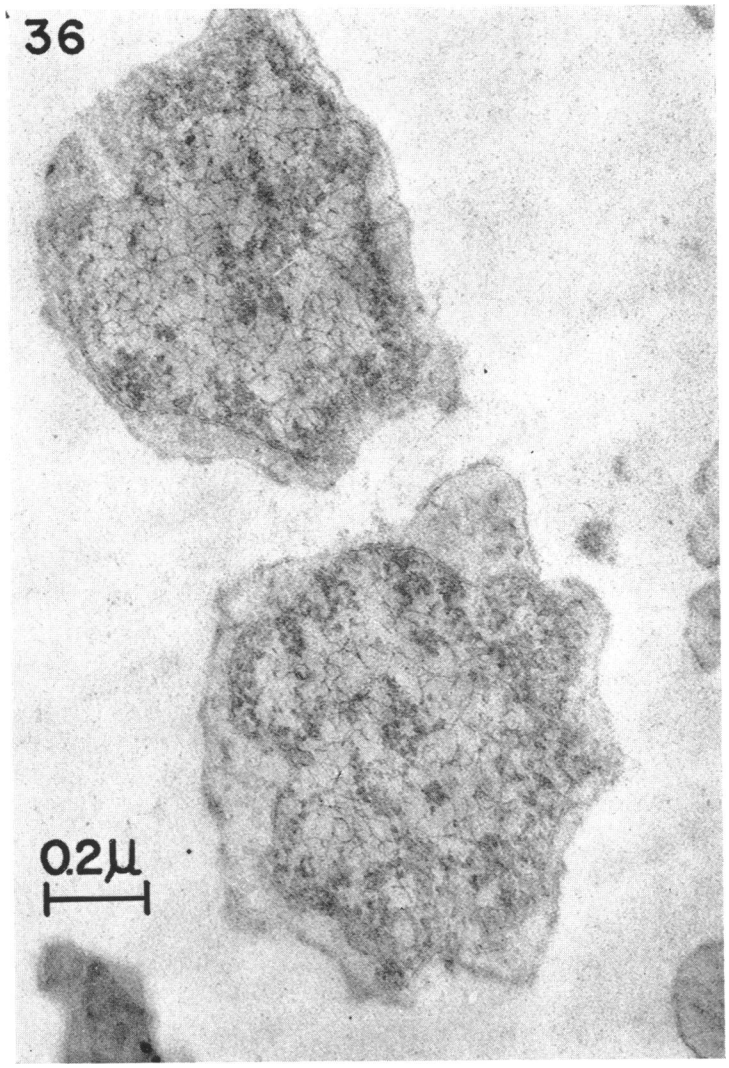


FIG. 36. *A thin section of strain 9b showing its procaryotic nature.*

branch, characteristics not reported for the stalks of caulobacters.

Our present work has disclosed a great diversity in the types of microbial cellular appendages (Fig. 2–11). There are variations in length as well as in width; some appendages taper to the distal tip, others do not; one has transverse striations; another bears buds; some may not contain cytoplasm.

Although the arms of strain 4a superficially resemble the *Caulobacter* stalk, annular rings have never been observed; the bifurcations are more suggestive of hyphal appendages which branch, a characteristic never reported for the stalk of caulobacters. This closer affinity to the hypha is supported by thin sections, which reveal that the appendages contain ribosomes and other cellular, nonmembranous material. The appendages of strain 4a are not completely analogous to hyphae because buds are never produced at the tips.

The appendages of strains 3a, 3b, and 9b are shorter than either stalks or hyphae. Their appearance is distinctive; they taper to a blunt tip. No membranous material has been found in them and they do not bear buds.

It is clear that the appendages of these new organisms are neither stalks nor hyphae. They include such a variety of structures that it is difficult to propose a single term to describe them all

TABLE 4. *Growth on complex nitrogen sources*

Medium	Optical density at 600 mμ		
	Strain 3a	Strain 4a	Strain 9b
Nutrient broth	0.022	0.018	0.048
YE medium	0.225	0.235	0.390
Standard growth media[a]	0.195	0.085	0.370

[a] Standard media: strains 3a and 9b, 0.02% GY; strain 4a, 0.01% YAmG.

TABLE 5. *Utilization of casein, Casitone, and Casamino Acids as sole sources of nitrogen and carbon*

Nitrogen source[a]	Optical density at 600 mμ		
	Strain 3a	Strain 4a	Strain 9b
Casamino Acids (0.1%)	0.035	0.020	0.105
Casitone (0.1%)	0.042	0.028	0.055
Casein (0.1%)	0	0	0

[a] The medium also contained 0.0005 M disodium phosphate, vitamins, and MS.

TABLE 6. *Utilization of inorganic sources of nitrogen*

Composition of medium[a]				Optical density at 600 mμ		
$(NH_4)_2SO_4$ (0.025%)	KNO_3 (0.025%)	Casamino Acids (0.01%)	Glucose (0.02%)	Strain 3a	Strain 4a	Strain 9b
−	−	−	−	0.005	0.005	0.005
+	−	−	−	0.005	0.003	0.005
+	−	−	+	0.252	0.028	0.030
−	−	+	−	0.008	0.010	0.018
−	−	+	+	0.158	0.031	0.168
+	−	+	+	0.262	0.028	0.130
−	+	−	−	0.005	0.002	0
−	+	−	+	0.010	0.005	0.010

[a] The medium also contained 0.001 M disodium phosphate, MS, and vitamins.

which would, at the same time, exclude stalks and hyphae.

There are two alternatives in naming these appendages Two names can be coined, one for the appendages of strain 4a, and another for the appendages of strains 3a, 3b, and 9b; or, one name can be introduced to include all cellular appendages of this type. The latter alternative seems preferable, in order to prevent the introduction of a plethora of terms to describe the appendages of each new organism. A single general term can serve (as do "flagellum" and "pilus") to define an important structure of the procaryotic cell.

The term *prostheca(e)* (from Greek meaning appendage) is proposed, with the following definition: *a semirigid appendage extending from a procaryotic cell, with a diameter which is always smaller than that of the mature cell, and which is bounded by the cell wall.* It is not a bud, although a bud may arise upon it.

By this definition, the cellular stalks of the

TABLE 7. *Carbon source utilization*

Carbon source	Utilization		
	Strains 3a and 3b	Strain 4a	Strain 9b
L-Arabinose	+	+	+
D-Ribose	+	+	+
D-Xylose	+	+	+
L-Fucose	+	+	+
L-Rhamnose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
D-Galactose	+	+	+
D-Lyxose	+	+	+
Maltose	+	+	+
Cellobiose	+	+	+
Lactose	+	+	+
Raffinose	−	−	−
Starch	−	−	−
Inulin (0.005%)	−	−	−
Pectin	−	−	−
Adipate	−	−	−
Phthalate	−	−	−
Benzoate	−	−	−
Methanol	−	−	−
Arabitol	−	−	−
Erythritol	−	−	−
D-Arabinose	+	+	?[a]
L-Sorbose	+	+	−
Melibiose	+	−	−
Melizitose	+	+	−
Arabin	+	+	−
Dextrin	+	+	−
Glycogen	+	−	−
Xylan	−	?	−
Formate	+	−	−
Propionate	+	−	?
Succinate	−	+	+
Fumarate	−	+	+
Butyrate	−	−	+
Ethyl alcohol (1%)	+	−	−
Glycerol	+	+	−
Adonitol	+	?	−
Dulcitol	+	−	−
Inositol	+	+	−
Mannitol	+	+	−
Sorbitol	+	+	−

[a] Not tested or utilization uncertain.

caulobacters and the hyphae of the hyphomicrobia are prosthecae, whereas the secreted stalks of *Gallionella* and *Nevskia* are not. Some flagella have been reported to be ensheathed by the cell envelope (R. J. Seidler and M. P. Starr, Bacteriol Proc., p. 42, 1967) but flagella are not prosthecae.

Nomenclature and taxonomy. The prosthecae of these new strains distinguish them from known bacteria, but the prosthecate forms do not comprise a completely homogeneous group. The isolated strains can be separated into three groups on the basis of morphological and physiological characteristics. The major differences among these groups have been listed in Table 3.

Strain 4a differs from the other isolates in morphology, physiology, and mode of reproduction. Not only are there fewer prosthecae per cell, but they are longer than those of forms 3 and 9. In addition, this is the only strain which can grow anaerobically. Finally, strain 4a reproduces by budding rather than by binary fission, which is characteristic of the other strains.

There are two families of known bacteria which resemble strain 4a. These are the *Caulobacteraceae* and *Hyphomicrobiaceae*. The caulobacters differ from strain 4a in having only one prostheca per cell, dividing by binary fission, having motile swarm cells, and possessing a membranous prosthecal core. The hyphomicrobia differ from strain 4a in having fewer prosthecae per cell, bearing buds on their prosthecae, and having motile swarm cells. Neither group is known to contain gas vacuoles.

These major differences allow a clear separation of strain 4a from all the known bacteria and justify the establishment of a new genus and species:

Ancalomicrobium adetum, gen. et sp. n.

Ancalo-, from Greek meaning arm; adetum, from Greek meaning unattached.

Unicellular, gram-negative bacteria having from two to eight prosthecae extending from the cell. These prosthecae attain a length of about 3 μ at maturity, approximately three times the diameter of the cell. The prosthecae may have bifurcations but do not themselves bear buds. Cells reproduce by budding; buds are formed at one position on the budding cell. As buds develop, two or more prosthecae differentiate from the bud. Division occurs transversely when the mother and daughter cells have attained approximately the same size. Cells are nonmotile and have no holdfasts. Cells may contain gas vacuoles. Colonies are circular in form, convex in elevation, with an entire margin. Euryoxic, ferments glucose, but does not reduce nitrate. Catalase present. Ammonium can be used as a sole source of nitrogen for growth. An organic energy and carbon source and panthothenic acid are required for growth. Table 7 lists the utilizable carbon sources. This is the type species. Strain 4a is the type strain.

In the Davis creek water, another organism has been observed which resembles this organism very closely (Fig. 7). This organism differs from *A. adetum* in that the bud is borne at the end of a prostheca. Approximately 50 specimens of this organism have been observed; in all cases, only one appendage bore a bud. Its overall appearance is suggestive of a tripod with three budless prosthecae as a base. At the time of division, two such tripods are present, the daughter cell being a mirror image of the mother.

The taxonomic treatment of groups 3 and 9 is more difficult. They show similarity to one another, but little obvious morphological similarity to other known bacteria. Some of the important characteristics differentiating strains of groups 3 and 9 are listed in Table 3. Group 3 strains have much longer prosthecae, and their cells contain gas vacuoles. Unlike the strains of group 9, the strains of group 3 are nonmotile and do not have flagella. In addition, strains 3a and 3b can utilize a large number of carbon sources as compared to strain 9b (Table 7). None of these differences is of itself important enough for the establishment of separate genera. Prosthecal length cannot be considered of major importance. Some members of the genus *Halobacterium* contain gas vacuoles and others do not. The presence of motile and nonmotile strains within a genus is known. In addition, the DNA base ratios are not sufficiently different to warrant the creation of separate genera. Because of these considerations, a single genus is proposed to contain groups 3 and 9.

Prosthecomicrobium, gen. n.

Prostheco-, from Greek meaning appendage.

Unicellular, gram-negative bacteria with prosthecae tapering to a blunt tip and extending in all directions from the cell. The ratio of prosthecal length to cell diameter (approximately 1.0 μ) is normally less than 1.0, although longer prosthecae are occasionally found. Cells divide by binary fission. Obligately aerobic, nonfermentative. May or may not contain gas vacuoles. May or may not be motile with a single subpolar to polar flagellum. May or may not be pigmented.

Two species are proposed for this new genus, one to include strains 3a and 3b, the other to contain strain 9b.

Prosthecomicrobium pneumaticum, gen. et sp. n.

Pneumaticum, from Greek meaning inflated.

The ratio of prosthecal length to cell diameter (ca. 1.0 μ) is approximately 1.0, although occa-

sionally much longer prosthecae are found. The cytoplasm contains distinctive spindle-shaped vesicles (ca. 100 × 300 mμ) comprising gas vacuoles. Nonmotile. Ammonium, but not nitrate, can be used as a sole source of nitrogen if appropriate vitamins and an organic energy and carbon source are included. Vitamins B_{12}, thiamine, and biotin are required for growth. Colonies are not pigmented. Table 7 lists the utilizable carbon sources. This is the type species for the genus. Strain 3a is the type strain.

Prosthecomicrobium enhydrum, gen. et sp. n.

Enhydrum, from Greek meaning aquatic.

The ratio of prosthecal length to cell diameter (ca. 1.0 μ) is less than 0.5. No gas vacuoles. Motile by a single, subpolar to polar flagellum. Ammonium, but not nitrate, can be used as a sole source of nitrogen if appropriate vitamins and an organic carbon source are included. Thiamine is required. Colonies may or may not be pigmented (yellow and red have been observed). Table 7 lists the utilizable carbon sources. Strain 9b is the type strain.

The cultures (strains 3a, 4a, and 9b) have been deposited in the American Type Culture Collection (ATCC), Rockville, Md., and in the International Collection of Phytopathogenic Bacteria (ICPB), Davis, Calif.

No higher taxonomic relationships are proposed at this time, as it is not clear where these new organisms should be placed. *Ancalomicrobium* could be assigned to the order of budding bacteria on the basis of its mode of division; but this would require the establishment of a new family or the redefinition of one now existing. It is not clear where *Prosthecomicrobium* should be placed, although, until now, all isolated bacteria known to contain gas vacuoles have been placed in the order *Pseudomonadales*. Another possibility would be to include all prosthecate bacteria in one order, which would contain the caulobacters, the hyphomicrobia, *Planctomyces*, *Pedomicrobium*, and the genera described here.

Nutrition and ecology. Freshwater contains materials capable of supporting growth of chemoorganotropic organisms (3). These materials are humic substances and substances excreted by the phytoplankton, which vary from simple organic acids, e.g., glycolic and oxalic (1, 35), to polysaccharides (20).

Prosthecomicrobium and *Ancalomicrobium* are chemoorganotrophs capable of using a variety of organic compounds. Monosaccharides and disaccharides are widely used by all, whereas benzoic and phthalic acids and most polysaccharides are not used. The sugar alcohols, sorbitol, mannitol, and inositol are used by *Ancalomicrobium* and *P. pneumaticum* but not by *P. enhydrum*, which has the least nutritional versatility of the strains studied.

All strains require vitamins. This is not unexpected, since many of the marine and freshwater algae have requirements for one or more of the following vitamins: B_{12}, thiamine, and biotin (26). More significantly, the caulobacters, typical freshwater bacteria, require vitamins (10, 24). Biotin, thiamine, and nicotinic acid have been found in freshwater by Hutchinson (17) and Hutchinson and Setlow (18).

In addition to the nutritional evidence, there are several other indications that the new forms are autochthonous freshwater bacteria. The prosthecae resemble the appendages of certain planktonic protists, e.g., *Scenedesmus*, *Chaetoceros*, and *Staurastrum*. Poindexter (24) has suggested that the stalks of the caulobacters and the projections of eucaryotic microorganisms have an essentially similar function, to reduce the rate of settling. On the other hand, Pate and Ordal (22) have proposed that, by increasing the surface membrane area, the stalk enables an organism to live in an environment low in nutrients. Either interpretation is in accord with the view that these new prosthecate bacteria are indigenous to water. The presence of gas vacuoles in *A. adetum* and *P. pneumaticum* also fits this postulate, since other procaryotic organisms known to contain gas vacuoles are typically aquatic.

ADDENDUM

After completing the manuscript, a recent paper by D. I. Nikitin and S. I. Kuznetsov (Microbiology **36**:934–941, in Russian) was brought to the author's attention. This article contains electron micrographs of microorganisms found in lake water. Three of these microorganisms (Fig. 17, 18, and 19) have multiple appendages; one (Fig. 19) is of form 3.

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