STUDIES ON A STRAIN OF CAULOBACTER FROM WATER

I. Isolation and Identification as *Caulobacter vibrioides* Henrici and Johnson with Emended Description

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This study started as an attempt to determine if well waters have a flora of attached bacteria comparable to that of surface waters and to cultivate any species that might be found. The isolation of one species has led to studies of its characteristics and recommendations on its taxonomy.

EXPERIMENTAL METHODS AND RESULTS

Occurrence of attached bacteria in well waters. Samples were collected from several wells using sterile glass jars of approximately one gal capacity. After the samples had been brought to the laboratory, they were examined for attached bacteria by the method of Henrici (1933). Slides, attached to rubber-covered wire, were suspended in the water, either in the original sample jars or in smaller jars containing 750 ml amounts of the sample. After various periods of time the slides were removed and examined, either after fixing and staining or as wet mount preparations. For the latter purpose a drop of rose bengal staining solution was placed in the water remaining on the slide immediately after it was withdrawn from the sample jar. A cover glass was placed then over the preparation and sealed to the slide with vaspar. A phase microscope was found to be better than a bright field microscope for the examination of the wet mounts.

Attached bacteria were seen on slides from water from every well. The results from one well will be described in some detail. Figure 1 shows a typical field on a slide that had been immersed for 12 days. Numerous stalked rods, varying in length from 2 to 6 μ, sometimes straight, but usually slightly curved, were seen. The width of the stalks was approximately 0.3 μ and the length varied from a barely noticeable extension of one end of the cell to approximately 15 μ. Holdfasts were evident at the ends of some of the stalks. Slides from this well water were examined after 24, 48, and 72 hours. On the 24 hr slide, attached bacteria were widely scattered. Of 50 bacteria counted, 9 had definite stalks. Many stalks were so short as to be barely perceptible; the longest was 1½ cell lengths. On the 48 hr slide, there were an increased number of cells and increased stalk formation. A random field contained 48 cells, of which 18 had definite stalks ranging up to 4 cell lengths; 3 had discernible holdfasts. A random field on the 72 hr slide contained 141 cells, of which 56 were stalked, with stalks ranging up to 7 cell lengths. Holdfasts were more common.

In wet mounts it could be observed that the stalks were attached to the slide and rose straight up from the slide. At the top of each stalk was a cell, and, as the fluid currents shifted, the cells and stalks waved much as tall grass in the wind, giving the impression that the stalks possessed considerable rigidity.

Attempts at enrichment of stalked bacteria. The following experiment was carried out on water from the well from which results have been reported, and a similar experiment was carried out with water from one other well. In each of 5 Coplin jars there were placed 60 ml of the water from the well and, in 5 other Coplin jars, 60 ml of a dilute broth containing 5 mg of peptone and 5 mg of yeast extract per liter. One jar of each series was used as a control, and enough $Na_2S \cdot 9H_2O$ was added to the other 4 jars to give percentages of 0.01, 0.005, 0.0025, and 0.00125. Each jar that contained dilute broth was inoculated with one ml of water from the well. Four slides were immersed in each jar, two being examined after 5 days and the others after

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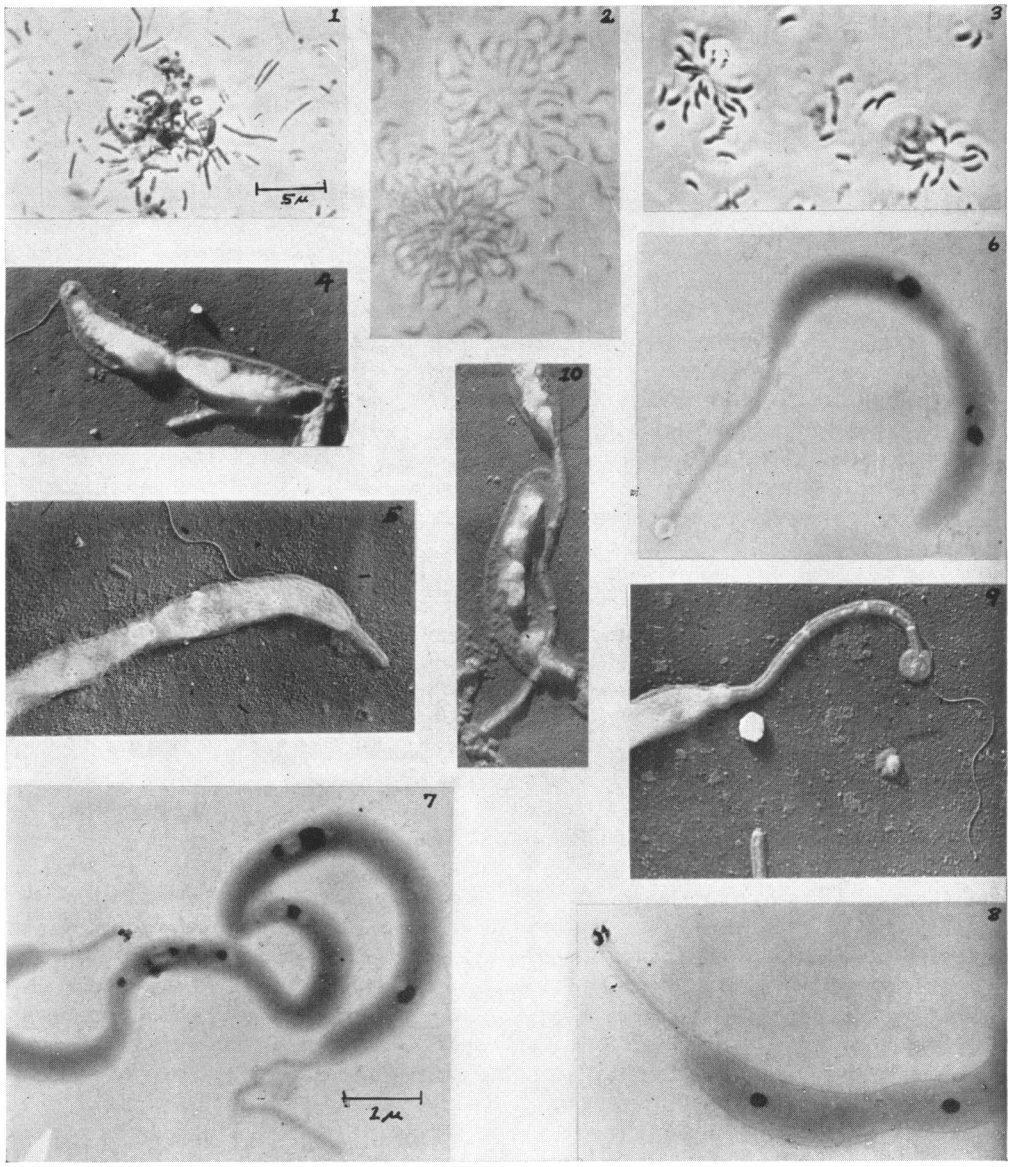

Figure 1. Attached bacteria with stalks and holdfasts, seen on slide that had been immersed in well water for 10 days. Stained with rose bengal. Scale applies to figures 1, 2, and 3.

Figures 2–3. Rosettes of *Caulobacter vibrioides*

Figure 2. From colony, stained with basic fuchsin.

Figure 3. From agar block culture, observed with phase microscope.

Figures 4–15. Micrographs of *Caulobacter vibrioides*. Scales in figures 7 and 15 apply to all micrographs. Figures 4, 5, and 9–15 shadowed with 135A chromium.

Figure 4. Late stage in transverse fission, parent cell stalked, new cell with flagellum apparently arising from inclusion.

Figure 5. Late state in transverse fission, stalk being formed on new cell previous to separation, dense inclusions partially melted by electron beam.

Figure 6. Stage in transverse fission, showing stalk, holdfast, dense inclusions, and cell sap vacuoles.

Figure 7. Filamentous form, showing stalk, holdfast containing dense material, cell sap vacuoles, and dense inclusions.

Figure 8. Stage in transverse fission, showing central filament of stalk, dense material in holdfast, and dense bodies.

Figures 9–10. Tubular stalks with central filament and walls which appear to be continuous with cell wall. In figure 10 cytoplasm appears to extend into stalk.

10 days. Less stalked bacteria were found with all percentages of sulfide. Approximately equal numbers were found on the slides in the well water control and those in the dilute broth control. Better developed and longer stalks, however, were found in the well water control.

Isolation and characterization of a stalked bacterium. When enrichment procedures failed to be of assistance in the isolation of the stalked bacteria, portions of the water were plated on nutrient agar, and numerous colonies were examined to determine if any of them consisted of stalked bacteria. One such colony was found. The following is a description of the characteristics of the culture isolated from this colony.

Cultural and biochemical characteristics. The organism grows well on most common culture media and is essentially heterotrophic. Nutritional studies, to be reported in a later publication, show that it requires riboflavin and an organic source of energy. The optimum growth temperature is approximately 30 C, with poorer growth occurring at 20 C and at 37 C. The pH growth range is from 6 to 9.

When first isolated the organism failed to grow on gelatin, but after adaptation to culture media, it produced surface growth and filiform growth along the line of puncture in gelatin stabs. Surface agar colonies are up to 5 mm in diameter after 96 hr, round, smooth, slightly raised, glistening, finely granular in the center, and grayish-white with the center and the reverse side becoming brownish-yellow. Subsurface colonies are dense, brownish-yellow, and lenticular, up to 0.5 mm in diameter and one mm in length. On agar slant cultures the growth is moderate in amount, filiform, grayish-white, glistening, and viscid. Moderate surface growth, with little or no growth in the stab, is produced in agar stabs. A moderate turbidity, with a slightly viscid sediment, is produced in nutrient broth. No detectable change in milk and little or no growth on potato are produced. Nitrates are not reduced to nitrites, and indole is not formed in tryptophan broth.

General morphology and staining reactions. Cytology. The morphology was determined from nutrient broth and nutrient agar cultures on preparations stained by the usual staining procedures. The developmental cycle of the organism was studied in nutrient broth films and in agar block cultures (Knaysi, 1940) using a phase microscope. Numerous cytological procedures were applied to the study of the organism using the light microscope. In addition, cytological studies were made with the electron microscope. Electron microscope preparations were made by placing drops of distilled water suspensions of the organisms on collodion films. The films were always supported by the deposition of 15 A of aluminum by vertical shadowing. In addition, some of the specimens were shadow-cast with approximately 135 A of chromium.

The cells are curved, vibrio shaped, ranging from 0.5 to 0.7 μ in diameter and 1.5 to 3.0 μ in length. Occasional filamentous forms are seen, especially in liquid medium cultures. Young cells are actively motile with a single polar flagellum. Older cells are attached by a polar stalk that is approximately 0.2 μ in width and may be any length up to about 5 μ. Holdfasts are frequently evident. A distinct slime layer or capsule is regularly present. It has been found to be readily stainable by Tyler's modification of Anthony's method (Anthony, 1931) and by Muir's method (Mackie and McCartney, 1942). The cells are gram negative.

Multiplication is by transverse binary fission (figures 4, 5, 6, and 8). The parent cell is stalked and usually attached. Before cell division is complete, a flagellum is formed at the free end of the new cell. It appears to arise from an inclusion in the end of the cell (figure 4). When division is complete, the new cell develops motion, breaks away from the parent cell, and becomes actively motile. A stalk is formed at the same end of the cell as the flagellum. In some cases the stalk starts to form before cell division is complete (figure 5). Usually it forms only after the cells have become motile and broken away. We have never been able to observe the exact relationship of the points of origin of the flagellum and the stalk, but they are at the same end of the cell and probably are close together (figures 5 and 9). The developmental cycle, as described, agrees with that observed by Houwink and van Iterson (1950) and Houwink (1951) with a similar or identical organism.

The stalk is an outgrowth of the cell and not a secretion. It appears to be made up of a central filament and a covering membrane that is continuous with the cell wall of the organism. The central filament is evident in the unshadowed preparation shown in figure 8 and the shadowed

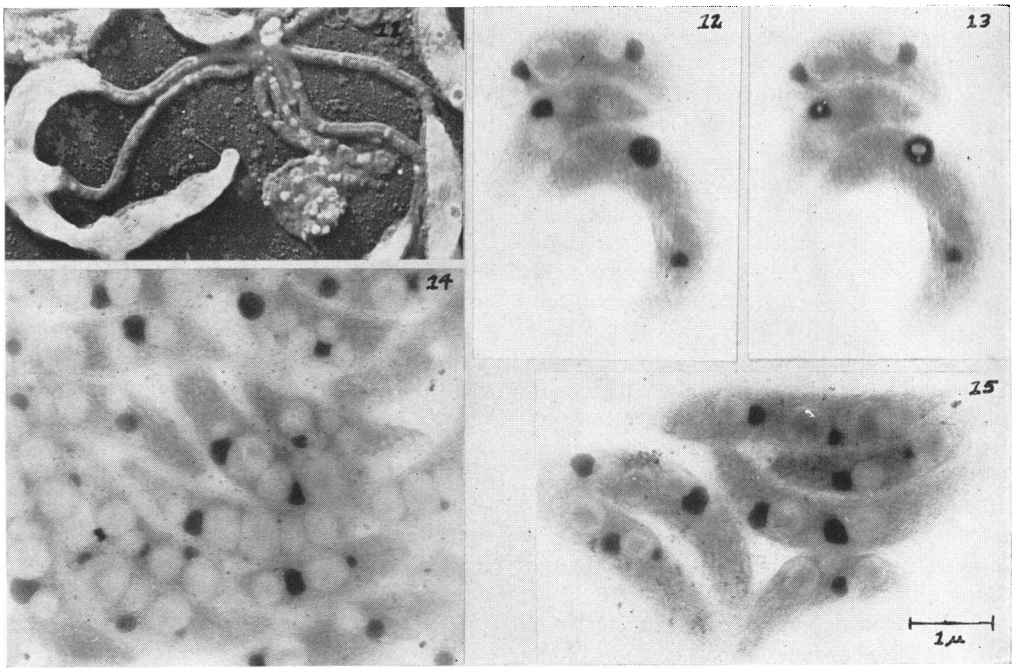

Figure 11. Portion of rosette.

Figures 12–13. In figure 13 dense inclusions have been melted by electron beam, micrograph taken two min after that in figure 12.

Figures 14–15. Cells from synthetic agar cultures. Cell sap vacuoles large and numerous with solid material deposited in them. Dense inclusions compressed by turgor of vacuoles.

Figure 14. 33 hr culture.

Figure 15. 131 hr culture with reduced numbers of inclusions and vacuoles.

preparations in figures 9 and 10. In figure 10 the cytoplasm of the cell appears to extend into the stalk. The covering membrane can be seen in figures 9 and 10. Houwink (1951) said that with his organism "the stalk is tube-shaped, and apparently contains protoplasm." Houwink describes the attachment of his organism to various *Bacillus* species in mixed culture. A few trials have failed to reveal such attachments with our organism.

Holdfasts may be seen in figures 6, 7, 8, 9, 10, and 11. One gets the impression that they are a secretion from the end of the tube shaped stalk. In the unshadowed preparations (figures 6, 7, and 8) materials that are dense to the electron beam may be seen in the holdfast. In the shadowed preparations, elevations in the holdfast suggest inclusions which may represent the same materials. In some preparations masses of more or less unformed substances which are similar in appearance to the substance of the holdfast are close to or connected with the holdfast.

The cells are arranged frequently in typical rosettes in which the stalks originate from a common point or region and the cells become arranged symmetrically around this point. Such rosettes were described and photographed by Jones (1905) with an organism that was probably identical with the one described here. The rosettes are especially numerous and large in preparations from agar colonies (figure 2). Similar rosettes have been seen on slides that had been immersed for several days in nutrient broth cultures. In preparations made directly from nutrient broth cultures and in agar block cultures, small groups that possibly represent rudimentary rosettes may be found. These are represented by figure 3, as seen with the phase microscope, and figure 11, as seen with the electron microscope. Hours of study with various types of microcultures, nutrient broth films, agar blocks, etc. have failed

to yield information on the mode of formation of the rosettes. Three possibilities suggest themselves: (1) Under certain conditions, stalks formed as in figure 5 may become attached, immediately, close to the attachment of the parent cell. (2) Some type of reproductive bodies may give rise to stalks upon which the mature organisms develop. (3) At the end of the motile stage, cells may group themselves by some sort of an agglutinative phenomenon. Virtually no evidence has been found to support any of these possibilities. In microcultures motile cells with stalks have been observed apparently to attach their stalks to the point of origin of stalks of a small group of cells. In almost every case, however, sometimes several minutes later, the cells may be seen to leave the group again.

Internally the cells show two types of inclusions that suggest the structure of mycobacteria as demonstrated by Knaysi *et al.* (1950) and Mudd *et al.* (1951).

One type of body, varying in diameter from 0.1 to 0.4 μ, is very dense to the electron beam. The other, varying in diameter from 0.2 to 0.6 μ, is less dense than the remainder of the cytoplasm. Both types of inclusions are more numerous and larger in cells that have been grown in a synthetic medium (to be described in a later publication) than in cells from nutrient agar (compare figure 14 with figures 6 and 7), and more numerous in younger cells (compare figures 14 and 15).

The dense bodies are spherical as seen in figure 8 where none of the less dense bodies is evident. In most cells they are distorted by pressure from the less dense bodies (figures 6, 7, 14, and 15). When the electron beam is applied to the cells, small light areas appear in the dark bodies. With higher magnifications these can be seen in all of the cells in preparations such as those shown in figures 6, 7, etc. These light areas appear to move as if floating on the surface of the dense body and coalesce together to form larger areas. Figure 13, a micrograph taken two minutes after that in figure 12, shows this phenomenon. It is evident also in shadowcast preparations (figures 5 and 11). This phenomenon has been called "melting" by Konig and Winkler (1948) and "volatilization" by Mudd *et al.* (1951). Mudd *et al.*, however, obtained volatilization only with a 200 kv beam whereas we have found it to occur with a 50 kv beam. Mudd *et al.* demonstrated that if the cells first were given exposure to a small amount of the electron beam, a "fixation" phenomenon occurred, protecting them against volatilization. We have noticed the same effect. Konig and Winkler considered their granules to be calcium phosphate in organic combination with nucleic acids, called "volutin" by various workers, while Mudd *et al.* considered their granules to be mitochondria.

The less dense bodies appear to be cell sap vacuoles. They are less dense than the cytoplasm and show pronounced turgor pressure as evidenced by the distortion of the dense bodies bordering on them. Figures 12, 13, 14, and 15 show indications of a membrane surrounding the vacuole and of the deposition of solid material in the vacuole. Whether this is an artifact of the preparation for electron microscope observation is not known. These observations on the cell sap vacuoles agree well with those of Knaysi *et al.* (1950) on the apparently corresponding structures of *Mycobacterium tuberculosis*.

The results of the application of many of the staining and histochemical procedures to this organism have been unsatisfactory due, probably, to the slime which surrounds the cells.

Lipoid inclusions have been demonstrated with Sudan III and by the method of Burdon (1946), using Sudan black B. Usually from 2 to 6 large inclusions are visible along with a variable number of randomly distributed small inclusions. It is probable that the larger inclusions are associated with the cell sap vacuoles that are seen readily with the electron microscope.

A reasonably good demonstration of the chromatinic bodies that have commonly been considered to be vesicular nuclei has been obtained with Smith's (1950) method using hydrolysis for 12 min at 60 C. With young cultures the number of chromatinic bodies varies from 2 to 8 per organism. The size and arrangement of these bodies indicate that they are not identical with the dense bodies seen with the electron microscope. Their association with the inclusions described as cell sap vacuoles has not been completely ruled out.

Taxonomy. Our organism obviously belongs in the *Caulobacteriaceae* Breed, Murray, and Hitchens and in *Caulobacter* Henrici and Johnson as included in *Bergey's Manual* (Breed *et al.*, 1948). The inclusion, however, will necessitate a change in the following sentence from the description of the suborder: "The stalked cells are asymmetrical in that gum, ferric hydroxide or

other material is secreted from one side or one end of the cell to form the stalk." As has been pointed out, the stalk appears to be a genuine extension of the cell and not a secretion.

Our strain is related to, or identical with, the organism described by Jones (1905). If it be considered that she mistakenly described the stalks as flagella, then the only differences in described characteristics are minor ones among the cultural characteristics. She did not propose a name for the organism. Omeliansky (1914) proposed the name *Bacillus flagellatus* for an organism of similar morphology, the "flagella" responsible for the species name probably being stalks. His description was incomplete since he was unable to obtain growth on transfer from the original colony. This failure to grow on common media makes the identity of the organism with ours and with that of Jones doubtful. Henrici and Johnson (1935) proposed the name *Caulobacter vibrioides* for an organism which they designated as the type species of *Caulobacter*. They rejected the name given by Omeliansky on the basis that it could not be established that his "flagella" were actually stalks. The description of Henrici and Johnson is also incomplete since it was drawn entirely from microscopic observation of organisms on slides that had been immersed in water. Since their description does agree with the characteristics of our organism, we are proposing that our organism be identified as a strain of *Caulobacter vibrioides* and that the description of the species be emended to include the characteristics that have been determined in this study.

Attention has already been called to the similarity of our organism to that of Houwink (1951).

SUMMARY

Attached bacteria have been demonstrated in samples of well water by the method proposed by Henrici in 1933. Enrichment of the samples by sulfide decreased the number of attached bacteria, and enrichment by peptone and yeast extract failed to have any effect on their numbers. A heterotrophic organism that was isolated on nutrient agar proved to be similar to, or identical with, an organism that was first described by Jones in 1905. The cells are gram negative, vibrio-like, with rounded ends, 0.5 to 0.7 μ in diameter and 1.5 to 3.0 μ in length; filaments are formed occasionally. A thick slime layer or capsule is present. Young cells are actively motile with a single polar flagellum. A stalk with a central filament that appears to be continuous with the cytoplasm and a wall that appears to be continuous with the cell wall later develop at the same end as the flagellum. The cells become attached with a holdfast, frequently in rosettes, on surfaces exposed in water or a liquid medium; larger rosettes appear on a solid medium. Multiplication is by transverse fission, the flagellum and sometimes a rudimentary stalk being formed before division is complete. Electron microscope studies have revealed two types of inclusions. One is dense to the electron beam but is "melted" or "volatilized" by it. The other is less dense than the cytoplasm and appears to be a cell sap vacuole. The cultural and biochemical characteristics of the culture have been described.

It is recommended that the culture be identified as *Caulobacter vibrioides* and that the description of this species be emended to include the characteristics that have been determined in this study.

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