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THE BACTERIAL FLORA OF DISTILLED AND STORED WATER. I. GENERAL OBSERVATIONS, TECHNIQUES AND ECOLOGY

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SUMMARY. A total of 36 samples of distilled water from 10 different sources were plated on salt-free agar, incubated at 20°C, and colonies of different appearance fished. Duplicate plates of selected samples were incubated at 37°C. Samples were also plated on agar with 1% NaCl added for study of incidence of psychrophils and halophobes. A number of plates were prepared from fresh river water, from such water stored in the laboratory for several weeks, and from distilled water to which a small amount of soil had been added. A few plates were also exposed directly to laboratory air. From the various plates were fished all colonies which appeared different; a total of 306 isolates was obtained. These cultures were studied morphologically, culturally and physiologically. The great majority of the distilled water isolates were Gram-negative, polar flagellated, nonfermentative straight rods. Sporeformers, cocci, typical spirilla, and fermentative bacteria were absent and the difference between the flora of distilled water on the one hand and river water and air on the other is striking. In addition to the usual types of bacteria, there were found several types of aggregating bacteria which are described in some detail.

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The distilled water in most laboratories ordinarily comes from storage tanks which are more or less open, and the air apparently carries with it and into the water sufficient

nutrients to support an extensive bacterial flora. This flora is more characteristically a pure water flora than that of ordinary lakes and rivers. In the latter are found a variety of bacteria, more or less transient, which developed elsewhere such as bottom mud, adjacent land or aquatic animal and plant life. For comparative purposes a number of isolates were made from fresh river water and such water stored in flasks for various lengths of time. Isolates were also obtained from stored distilled water to which had been added a small amount of garden soil. Agar plates of the same composition as for culturing the distilled and stored waters were exposed directly to outside air and representative colonies cultured. Although it is most likely that the majority of the bacteria in a sample of distilled water originated from the surrounding air the data presented show that only a small percentage of common air bacteria survive or multiply in distilled water. Typical soil bacteria such as sporeformers and other Gram-positive rods, and cocci are rare in distilled water.

Materials and Methods

The distilled water samples were obtained from taps in laboratories having a central supply. Samples from the following locations were studied: 1) author's laboratory, Chicago, 2) Chicago Medical School, 3) Veterans Hospital, Chicago, 4) Illinois State Health Laboratory, Chicago, 5) University of California, Davis (courtesy of M. P. Starr), 6) Fisheries Research Laboratory, Vancouver, B.C. (courtesy R.A. MacLeod), 7) National Institute of Genetics, Japan (courtesy Tetsuo Iino), 8) George Washington University, Washington, D.C. (courtesy R. Hugh), 9) British Vinegars Ltd., England (courtesy J.L. Shimwell), 10) Department of Agriculture, New Zealand (courtesy J.D. Stout). From most of the sources were obtained several samples, a total of 36. The samples were plated when received and some again after storage in the refrigerator for about two months.

The plating medium used was originally developed for the culture of Caulobacter and is probably as good as any single medium could be for the purpose: Peptone (Casitone, Difco) 0.3%, yeast extract 0.1%, K_2HPO_4 0.1%, agar 1.5%, pH 7.1. The poured agar plates were dried at 37°C for one day and 0.1 ml of the diluted sample spread over the plate. The

dilutions were made in sterile distilled water. The inoculated plates were incubated at 20°C for three to five days and all colonies of different appearance were fished. In some instances duplicate plates were made with 1% of NaCl added to the medium to determine the incidence of halophobic* organisms. Some duplicate plates were also incubated at 37°C to determine the incidence of psychrophilic** organisms. The colonies picked from the plates were cultured in broth of the same composition as the plating medium without agar. This was the basic broth used throughout the study.

The morphology was determined by Gram stain and flagella stain (Leifson 1951, 1960), halophobism or osmophobism by culturing in the basic medium with 1% NaCl added. This concentration of salt was chosen because it inhibited the growth of Caulobacter vibrioides which may be regarded as a typical halophobe. Hydrolysis of gelatin was determined in the basic broth with 8% gelatin added. Deep tubes were inoculated and incubated at 20°C for up to 10 days. Reduction of nitrate was determined in the basic broth with 0.2 % KNO_3 added. An inverted vial was placed in each tube to trap any gas produced. Incidentally it was found that the gelatin and nitrate tests could be performed in a single gelatin-nitrate medium made by adding 8% gelatin and 0.2% KNO_3 to the basic broth. The nitrate does not appear to influence gelatin liquefaction nor does the gelatin affect nitrate reduction. To insure that any gas produced from the nitrate is trapped as bubbles the medium should be inoculated while liquid or allowed to melt before being placed in the 20°C incubator. Carbohydrate utilization was determined primarily in the oxidation-fermentation (O-F) medium of Hugh and Leifson (1953) modified by omitting the NaCl. The carbohydrates were autoclaved separately in 10% aqueous solution and added aseptically to the sterile base. As an additional check on the utilization of carbohydrates and to determine any accessory growth factor requirements, two

*A halophobe is here defined as any organism which fails to produce macroscopically visible growth in a medium having an osmotic pressure equivalent to 1% (w/v) of NaCl.

**A psychrophil is here defined as any bacterium which fails to produce macroscopically visible growth when incubated at 37°C.

other basic media were used. The first of these was entirely synthetic with the following composition: NH_4Cl 0.1%, Na_2SO_4 0.02%, K_2HPO_4 0.02%, $MgCl_2 \cdot 6H_2O$ 0.02%, $CaCl_2$ 0.005%, pH 7.1. The second medium had the same composition with the addition of 0.002% yeast extract. To the sterile media was added aseptically a sterile solution of the carbohydrate to give a concentration of 1% as in the O-F medium. Without the carbohydrate, medium number 2 supported only a trace of macroscopic growth and medium number 1 obviously none. After incubation for five days at 20°C a test for acidity was made by the addition of brom thymol blue. The Casamino Acids medium was made by adding 0.2% vitamin-free Casamino Acids (Difco) to distilled water, pH 6.9-7.1. Catalase was determined by the addition of hydrogen peroxide to broth cultures. Slants of the basic plating medium with 0.2% soluble starch added were employed for determining starch hydrolysis. When good growth was observed a drop of Gram's iodine solution was added to the slant. Cellulose hydrolysis was determined by placing a strip of filter paper in the broth. Several oxidase tests were tried, but none were sufficiently satisfactory to justify a report. Milk was used at first but found to be rather unsatisfactory, hence no report.

Influence of Temperature and Salt Concentration on Colony Counts

From each of 8 of the distilled water samples were prepared plates for colony counts on the salt-free agar and on the 1% NaCl agar with incubation temperatures of 20°C and 37°C. Table 1 shows a summary of the colony counts.

Table 1. Effect of salt concentration and temperature on colony counts (the figures represent mean relative number of colonies per ml).

	% NaCl concentration		
Temperature	0	1	Halophobes
20°C	100	35	65%
37°C	70	15	80%
Psychrophils	30%	55%	

In the salt-free medium at 20°C the colony counts ranged from 500,000 to 2,000,000 per ml. The sample from each specimen showed a higher count in the salt-free medium than in the 1% NaCl medium. On the good assumption that distilled water contains neither thermophils nor halophils the percentages of halophobes and psychrophils were calculated and recorded in Table 1. The effect of combined high temperature and high salt concentration is striking. The colony count on the 1% NaCl agar incubated at 37°C was only 15% of that on the salt-free agar incubated at 20°C. This indicates that perhaps 85% of the colonies on the salt-free agar at 20°C consisted of bacteria which were either psychrophilic, halophobic, or both.

The above data show that temperature and salt relationships are not independent. An organism may grow well in a salt-free medium at both 20°C and 37°C and in a 1% NaCl medium at 20°C but not at 37°C. Such an organism would thus be a halophobe at 37°C but not at 20°C. At 20°C an organism may grow well in both the salt-free and 1% NaCl medium but at 37°C show growth only in the salt-free medium. Such an organism would be a psychrophil when grown in the 1% NaCl medium but not when grown in the salt-free medium. A limited study of a few of the halophobes isolated showed comparable inhibiting effects by salts other than NaCl, and by nonionic substances such as glucose. A better term for these bacteria would be osmophobes or osmophobic bacteria.

Effect of pH on Colony Counts in Salt-Free and 1% NaCl Agar

The effect of pH on colony count was studied using one sample of distilled water and 20°C incubation. This rather limited study is summarized in Table 2. It is evident from the table that the distilled water sample contained few acidophilic bacteria. The optimum pH was closer to 7 than to 5 or to 9. The reduction of colony count on the 1% NaCl agar as compared with the salt-free agar is much greater at pH 9 than at pH 7. These limited data show that an organism may be more halophobic at one pH than at another.

Table 2. Effect of pH of plating medium on colony counts at 20°C (the figures represent mean relative counts).

pH	% NaCl concentration 0	1	Halophobes
5	1.2	0.6	50%
7	100	50	50%
9	77	10	87%

The Flora of Distilled Water Compared with that of Natural Water and Air

In the water from rivers and lakes it is common experience to find many fermentative types of bacteria of the coliform group, Serratia, Chromobacterium, Aeromonas, etc. Strains of these genera were not isolated in a single instance from any of the distilled water samples. Nor were there isolated any sporeformers and but few Gram-positive bacteria. No cocci of any kind were isolated. Typical spirilla were also absent. The predominant bacteria of the distilled water were rod shaped, Gram-negative, nonfermentative and polar flagellate. The main exception was a group of nonfermentative, peritrichous flagellate rods, some of which stained weakly Gram-positive and others Gram-negative. In Table 3 is recorded the flagellation of the organisms isolated and retained for detailed study. The number of organisms isolated from air was very small. It should be noted that the figures in the table do not show the incidence of flagellar types in the water itself but only in the isolated cultures.

Table 3. Incidence of flagellar types in isolated organisms (figures in parentheses are the actual number of cultures studied).

Source	Types of Flagellation Polar	Peritrichous	Atrichous	Total
Air	12%(1)	33%(3)	55%(5)	100%(9)
Stored water	69%(38)	20%(11)	11%(6)	100%(55)
Dist. water	80%(158)	13%(25)	7%(13)	100%(196)

If we assume the distilled water flora to originate mainly from air the change on storage of the water is very striking. The nonflagellate and peritrichous flagellate flora of air, dust, etc. is largely replaced in the water by polar flagellate types.

General Nature of the Distilled and Stored Water Flora

Originally 308 cultures were isolated but this number was reduced to 260 by the elimination of cultures from the same samples which obviously appeared identical. These 260 cultures were studied as outlined under Materials and Methods.

Temperature and Osmotic Relationships

The temperature relationships were determined using the salt-free medium and incubation temperatures of 20°C and 37°C and the osmotic relationships in the salt-free and 1% NaCl media incubated at 20°C. Selected cultures were also tested in media with 0, 0.5, 1.0 and 2.0% NaCl and incubated at both 20°C and 37°C.

Neither the term psychrophil nor halophobe is very specific, being interdependent to some extent and very much dependent upon the time factor. With a definite medium, incubation time and inoculum, an organism may be labeled as psychrophilic if macroscopically visible growth is absent when incubated at 37°C for a time which gives good growth when incubated at 20°C. Some of these cultures without macroscopic evidence of growth may show microscopic growth, and upon prolonged incubation may show macroscopic growth as well. The same considerations hold for the term halophobe. Psychrophilism and halophobism are of great practical importance from the standpoint of isolation and cultivation of an organism but of limited taxonomic importance. Both characteristics appear to reflect the recent ecology of the bacteria and often fail to correlate with characteristics of a more definite nature. Several groups of bacteria were isolated from distilled water which were quite homogeneous morphologically, tinctorially and physiologically but quite heterogeneous as to growth rates at various temperatures and salt concentrations.

Psychrophilic bacteria are abundant in nature and their presence is often overlooked because of the common practice of adding salt to culture media. Subinhibitive salt concen-

tration and incubation temperature often showed striking morphological effects. Several different types of halophobes developed almost endless filaments in media with 0.5% NaCl (see Figs. 15, 16). With others best filament formation was produced in media with 1% NaCl. Many of the psychrophils developed filaments when incubated at temperatures close to the maximum. Bacteria which are both psychrophilic and halophobic may show best filament formation when both salt concentration and temperature are elevated above the optimum. When incubated at 37°C many of the psychrophilic cultures were not only inhibited but rapidly killed. Some of the cultures, labeled psychrophilic because macroscopic growth was not apparent after incubation at 37°C, showed macroscopic growth when subsequently transferred to 20°C incubation. Most, if not all, of this latter type showed microscopic evidence of growth at 37°C, usually in the form of long filaments. Cell division appears to be more sensitive to elevated temperatures and ceases at temperatures which still allows cell growth. Incubation of the halophobes in an inhibitive 1% NaCl medium for 24 hours at 20°C did not show any lethal effect. On removal of the salt, growth invariably resulted.

Gelatin, Nitrate and Catalase

These are generally satisfactory tests. Gas from nitrate is very characteristic of Pseudomonas aeruginosa and a few of the other Pseudomonas species but uncommon with other bacteria. Of the distilled water isolates 46% reduced nitrate; 18% to nitrite only and 28% to nitrogen gas.

The physical appearance of the zone of liquefaction in the gelatin was much the same for all the liquefying cultures, differing only in the extent of the liquefaction. The gelatin cultures were incubated at 20°C and final readings made on the tenth day. Further incubation is complicated by evaporation and does not seem to be justified. Since all the cultures studied were aerobic the liquefaction was confined to the surface. Most of the positive cultures showed only weak liquefaction and in only a few instances did the zone of liquefaction extend more than 1 cm below the surface. Nutrient gelatin appears to be a good medium in which to observe pigment production, often showing better pigmentation than agar slants. Of the distilled water isolates 29% produced gelatinase to a greater or lesser degree.

The catalase test, taxonomically, may be less significant than either the gelatin or nitrate tests. One factor of importance in performing the test is the age of the culture. Relatively old cultures, 1-2 weeks, may give a strongly positive test, while relatively young cultures, 1-2 days, may be negative. Of all the cultures studied, slightly less than 50% produced catalase at one age or another.

Accessory Growth Factor Requirements

With bacteria which show few positive physiological reactions the accessory growth factor requirements may be of considerable differential importance. A 0.2% solution of amino acids, such as vitamin-free Casamino Acids (Difco), may be used to show the need for various growth factors. Of all the cultures studied about 25% failed to grow in the Casamino Acids solution indicating inability to synthesize growth factors other than amino acids. With bacteria which can metabolize glucose, a synthetic salts medium plus glucose may be used to show the requirement for certain amino acids as well as for other growth factors. In this medium there is generally a positive correlation between acidity and growth but two of the cultures tested showed turbidity but no acidity. Evidently these organisms metabolized glucose without producing detectable acidity. Organisms which metabolize glucose and can grow in the Casamino Acids but which cannot grow in the mineral salts medium plus glucose must have a requirement for amino acids. Of the cultures tested about 20% had such amino acid requirements.

Carbohydrate Utilization

Starch and cellulose were not hydrolyzed by any of the distilled water isolates nor did any of these cultures ferment any of the carbohydrates tested. The O-F medium of Hugh and Leifson (1953) was prepared without salt and was generally satisfactory for determining carbohydrate oxidation. A majority of the cultures oxidized one or more of the carbohydrates tested but many were entirely negative and some were equivocal. A fair number of cultures oxidized sucrose or maltose but not glucose. It is important to keep this in mind and not label a culture as carbohydrate negative because it fails to oxidize glucose.

Most bacteria produce an alkaline reaction in peptone media and when the acid produced from the oxidation of the

carbohydrate is small the reduction of pH may be barely noticeable, if at all. With bacteria which do not require accessory growth factors, a simple synthetic base medium without an energy source may be more satisfactory than the O-F medium for determining carbohydrate utilization. If growth takes place in this medium the carbohydrate must serve as a source of energy. Usually, but not invariably, acid is produced if the carbohydrate is utilized. With bacteria which have accessory growth factor requirements these can usually be met by the addition to the synthetic base of 0.002% yeast extract. This medium by itself did not show more than the faintest trace of macroscopic growth. When the O-F medium shows a very weak or indefinite acid reaction the synthetic medium, with or without yeast extract, may give a more definite indication of carbohydrate utilization.

Of the distilled water isolates 14% failed to oxidize any of the carbohydrates tested, 83% oxidized glucose, 68% d-mannose, 67% maltose, 42% sucrose, 18% d-sorbitol, 12% raffinose. Of the glucose oxidizers 40% required an accessory growth factor of one kind or another.

Pigmentation

Aside from the water soluble greenish pigment of pseudomonads, water soluble pigmentation was not observed in any of the cultures. Of the water insoluble pigments yellow and pink were the most common. Several types of bacteria with yellow pigmentation were isolated. Most of these showed polar monotrichous flagellation. Among the few nonflagellate strains were three with huge capsules. The most common yellow organism was a polar flagellate which aggregated into characteristic rosettes. Ten isolates (possibly corynebacteria) produced a light yellow, or greenish-yellow, water-insoluble pigment. A pink type of polar flagellate was widely distributed. Several brick-red cultures and cultures with shades of orange, ochre and brown were isolated but only from the stored soil-water mixtures. Red pigmented Serratia and purple pigmented Chromobacterium were never seen. The orange and ochre cultures formed typical aggregates. Several of the cultures produced melanins on prolonged incubation. Among these were Caulobacter vibrioides and various pseudomonads.

Somatic Aggregation

The grouping or aggregation of bacteria in nature into distinctive patterns may be quite common: From the distilled and stored waters were isolated four different types of aggregating bacteria. Among these is Caulobacter vibrioides, which is a typical distilled water organism. C. vibrioides differed from the others by forming stalks and being unmistakably epiphytic. The other three aggregating types did not produce demonstrable stalks and convincing evidence that they were epiphytic was not obtained. How the aggregates are formed is still unknown. A typical aggregate in the form of a rosette when stained with a basic dye showed a hollow or empty center. Stained with the flagella stain this center appeared solid. This would indicate the presence of a nonstainable substance such as a polysaccharide which may hold the individuals in the pattern. Individual organisms stained with the flagella stain often showed some ragged amorphous material at one end of the soma as if the organism had been torn away after having been attached. In some flagella stains a thin strand of material is evident between individual bacteria indicating perhaps incomplete separation. These connecting strands may be the cause of the aggregation.

Morphology

The great majority of the bacteria isolated were small or medium sized, straight Gram-negative rods with polar monotrichous or multitrichous flagella. All types of flagella were represented from the tiny waves of Pseudomonas diminuta, with wavelengths of about 0.8 μ, to very long waves of 3 μ or more. With a few of the cultures the flagella were formalin sensitive and became straight or coiled as a result of the formalin treatment. The normal-curly flagellar variation was not observed in the polar flagellates but only in the peritrichous flagellates. The flagella stained without difficulty in all but a few of the cultures. Faint staining with the Leifson flagella stain, as previously observed with the polar flagella of some strains of Chromobacterium, was observed with a few of the polar flagellates. Among the 25 isolates with peritrichous flagella were types which could be classified as Alcaligenes, Achromobacter, Agrobacterium, Flavobacterium and Corynebacterium. Only the latter group was characteristic of distilled water and had extraordinarily

long flagella, 1-2 per organism, with both normal and curly waves.

Only a few of the isolates were definitely capsulated. The most striking in this respect were three strains of a large, yellow pigmented, atrichous organism isolated from the Chicago and California samples. From the Vancouver and Japanese samples were isolated six strains with lanceolate-shaped soma and similar single polar flagella. The soma of the three Japanese strains was distinctly larger than that of the Vancouver strains. A slight somatic curvature was observed in many of the isolates but probably has little taxonomic significance. With a single exception, typical spirilla or vibrio types were not isolated. From one of the California samples was isolated a tiny curved organism with a single polar flagellum. In the filamentous form it appeared like a spirillum or a spirochaete. This organism grew so slowly and produced such tiny and inconspicuous colonies that its presence in the water could easily be overlooked. However, it would not appear to be a typical distilled water type. Cocci, sporeformers, and typically filamentous forms were not isolated and apparently are rare in distilled water.

Taxonomy of Distilled Water Flora

In the classification of bacteria a choice has to be made regarding the relative importance of the various observable characteristics or else give equal rank to all characteristics. The latter choice seems to the author to be neither practical nor fruitful. Among the 300 odd cultures isolated from the distilled water samples and stored waters were some 50 different bacteria. Had more characteristics been determined further differentiation would undoubtedly have resulted. Bacteria which appeared to be of the same species were seldom identical unless isolated from the same sample of water. As a basis for classification the following characteristics are judged to be arranged in order of importance: 1) somatic shape, 2) autotrophism, 3) spore formation, 4) flagellar arrangement, 5) Gram reaction, 6) pigmentation (water insoluble), 7) nature of carbohydrate metabolism, and 8) individual physiological reactions. Considering the nature of the cultures in question, only 1, 4, 5, 6, 7, and 8 are pertinent. Of these, 1, 4 and 5 generally determine the family level; 6 and 7 the genus level; and 8 the species level. On this basis there are five families represented in the

distilled water flora: Spirillaceae, Achromobacteraceae, Corynebacteriaceae, Caulobacteraceae and Pseudomonadaceae. The Spirillaceae is represented by a single culture and this family is rare in the distilled water flora. Only 15 cultures could be classified in the family Achromobacteraceae, and of these only five originated from distilled water. Obviously this family is poorly represented in distilled water. One new species is tentatively included in the family Corynebacteriaceae. It was represented by ten cultures, nine of which originated from distilled water. These cultures were sufficiently alike to be included in a single species. Since they were isolated from four different sources (Chicago, Washington, England and New Zealand) they may be regarded as fairly typical of distilled water. The family Caulobacteraceae appears to be ubiquitous in distilled water. All of the cultures isolated were practically identical and could be labeled Caulobacter vibrioides. Most of the cultures isolated could be placed in the family Pseudomonadaceae.

The Aggregating Bacteria

The taxonomic significance of the phenomenon of somatic aggregation is, as yet, not clear. Bacteria which aggregate into definite patterns appear to be common, not only in distilled water but also in both fresh and salt water. Cultures similar to Caulobacter vibrioides in every respect except for stalk formation have been isolated from water. C. vibrioides itself in rich media tends to produce very short or no stalks at all. The phenomenon of aggregation may be of greater taxonomic significance than stalk formation and further study may show the logic of classifying the aggregating bacteria, including Caulobacter, in a separate and distinct family. No such taxonomic proposal will be made at this time. Below are described three different types of aggregating bacteria isolated from distilled and river water.

Xanthomonas and aggregating yellow types. Colonies of various shades of yellow were found on the plates from all the samples of distilled water, varying from bright yellow through yellowish-brown, orange, ochre to definitely brown. The bright yellow types were most characteristic of distilled water and the brown the least. The brown colonies were seen mainly on the plates from the stored soil-water mixtures and are more typically soil types of bacteria than water types. From the yellow colonies were isolated 45 cultures.

Of these one was peritrichous flagellate, four nonflagellate and 40 polar flagellate. The peritrichous flagellate had all essential characteristics of a species of Flavobacterium. Of the four nonflagellate cultures three showed huge capsules.

Among the 40 polar flagellate cultures were seven which did not aggregate and were characteristic of the genus Xanthomonas. Since only two of these were isolated from distilled water typical Xanthomonades are not characteristic of distilled water flora. The morphology of strain 170 from soil-water mixture, and of strain 227 from the Washington sample of distilled water is illustrated in Figures 12 and 13, respectively. The remaining 33 yellow cultures formed a rather homogeneous group and showed the phenomenon of somatic aggregation. Typical strains of this group were isolated from all sources of distilled water except the Japanese. The major distinguishing characteristics of the group is the yellow water-insoluble pigmentation, polar monotrichous flagellation, oxidative metabolism of carbohydrates and the somatic aggregation into definite patterns (Figs. 9-11). Distinct stalks have not been observed. There is no indication that the individual organisms at first swim around, as with C. vibrioides, and later attach themselves to each other. No evidence has been found of attachment to other bacteria. Apparently they are not epiphytic. Except for the absence of definite stalks the aggregates may resemble those of C. vibrioides. In addition to the simple rosettes, elongated groupings may form which appear as a chain of cells with lateral cells singly and in clusters. The flagellation is relatively poor with only a small proportion of the cells showing flagella. The flagellar wavelength is variable ranging from 1.6 to 2.4 μ in formalin fixed preparations. The physiological reactions of representative strains are recorded in Table 4. The majority of the strains were psychrophilic but several grew at 37°C. Where growth occurred at 37°C the organisms tended to develop into long filaments (see Fig. 16). All strains were more or less halophobic with only a few strains showing any macroscopic evidence of growth in the 1% NaCl medium. Where growth in the salt medium took place, submacroscopic or macroscopic, long filaments were usually observed. Filament formation appeared to be a characteristic reaction to unfavorable incubation temperature and unfavorable osmotic relations.

Other aggregating types. Two other aggregating types of bacteria were isolated. From river water were isolated three cultures which turned out to be very similar. The original agar colonies were recorded as orange, ochre and brown, respectively. The color of the brown colony was probably due to melanin, which this strain was later shown to produce, since young cultures were colorless. Morphologically the three cultures were very similar: small Gram-negative rods with a single polar flagellum of wavelength averaging 0.8-0.9 μ (Figs. 6, 7, 8). In broth cultures were formed small and large rosettes but no stalks. Except for being smaller and without the somatic curvature the morphological resemblance of these organisms to C. vibrioides is very striking. The organisms were psychrophilic but not halophobic which is the reverse of C. vibrioides. The physiological reactions are recorded in Table 4.

The other aggregating type is represented by a single isolate (strain 102) from a Chicago sample of distilled water. This organism is a nonpigmented, small Gram-negative rod with a single polar flagellum having an average wavelength of 1.3 μ (Figs. 1-5). The organism is psychrophilic but not halophobic. Rosette formation is very pronounced but stalks have not been observed. The physiological reactions are recorded in Table 4.

REFERENCES

Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. Jour. Bact. 66:24-26.

Leifson, E. 1951. Staining, shape and arrangement of bacterial flagella. Jour. Bact. 62:377-389.

_____. 1960. Atlas of Bacterial Flagellation. Academic Press, New York; London.

PLATE I

Figures 1 - 5. Aggregating type, nonpigmented. Strain 102.

1. Typical single individual.
2. Small rosette of four organisms. Note the dark center.
3. Large and medium rosettes.
4. Rosettes stained with basic fuchsin and not with flagella stain. Note the empty appearing centers of the rosettes as contrasted with the solid centers in the flagella stained preparations.
5. Large aggregate of rosettes.

Figures 6 - 8. Aggregating type, ochre or orange pigmented. Strain 6.

6. Typical single individual. The soma of this organism is somewhat plumper than that of Caulobacter vibrioides and does not have the characteristic slight curvature of the latter. The flagella of the two are similar.
7. The significance of the flagellum originating at the junction of the cells is not clear. In bacteria in general the flagella originate at the distal poles of a dividing form. The picture may represent an aggregate of three individuals which became attached at the flagellated ends as with caulobacter.
8. A medium sized aggregate. The upper organism has divided and the daughter cell developed a full length flagellum. The rosettes formed by this organism are very dense and any flagella originating from the points of attachment would be difficult to demonstrate.

Figures 9 - 11. Aggregating type, yellow pigmented.

9. Strain 10. Typical single individual with normal flagellum.
10. Strain 297. Cluster of rosettes. Three short, coiled flagella may be seen. Flagella have not been observed originating from center of rosettes or from points of attachment.
11. Strain 297. Small aggregate. Flagellum at distal pole of daughter cell but none at points of attachment of parent cells. Flagellation, however, was poor.

Figures 12 - 14. Xanthomonas species. Strains 170, 227 and 243 respectively. These strains showed little if any aggregating tendency.

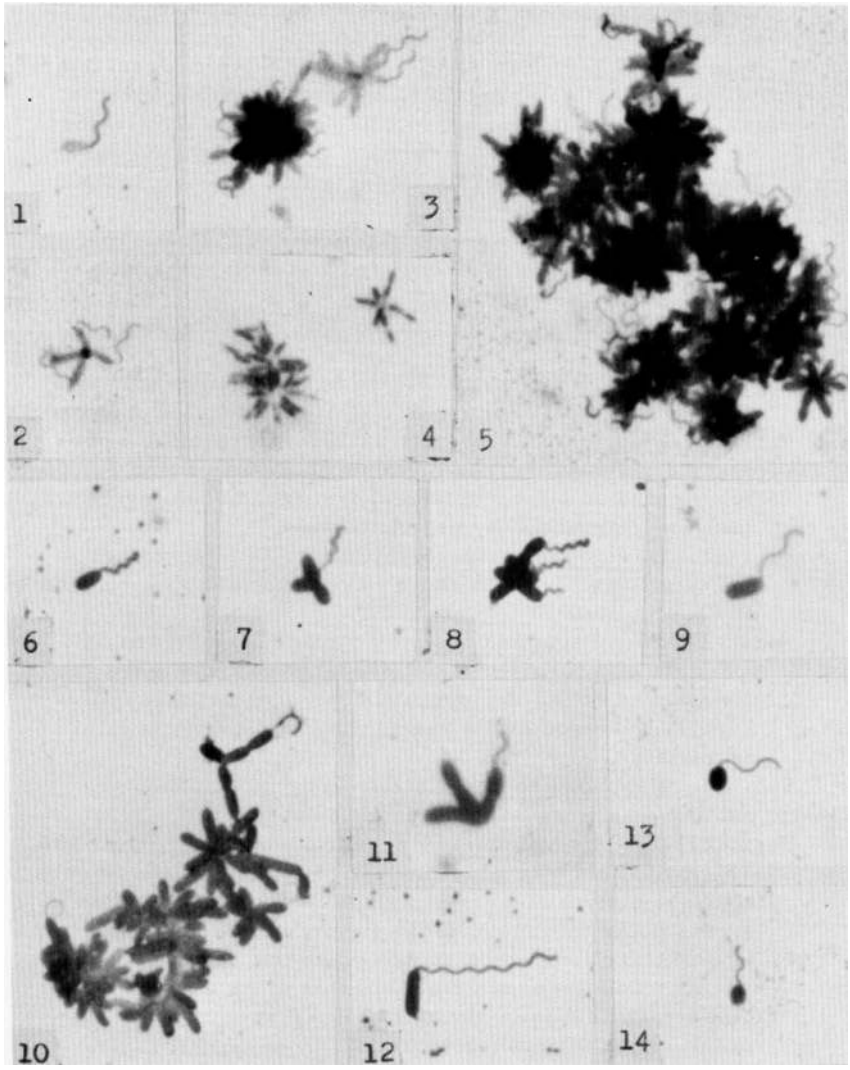


Plate I. Photomicrographs x 2,000. Leifson flagella stain.

PLATE II

Figs. 15, 16. Aggregating type, yellow pigmented. Strain 234. Figure 15 shows the normal morphology in a salt-free medium. Figure 16 shows what happens to the morphology when the organism is grown in a medium with from 0.5 to 1.0% NaCl. This is a typical reaction to elevated osmotic pressure by the halophobic strains.

Figs. 17, 18. Pseudomonas sp., pink pigmented. Strains 44 and 71 respectively. Various specific epithets have been proposed for pseudomonads which produce a water insoluble pink pigment.

Fig. 19. Pseudomonas sp., red pigmented. Strain 164. This was a beautifully flagellated rod isolated from a stored soil-water mixture.

Fig. 20. Pseudomonas sp. Strain 75. Note the distinct capsule.

Fig. 21. Pseudomonas sp. Strain 84. The light staining soma was typical of many of the distilled water bacteria.

Fig. 22. Pseudomonas sp. Strain 175. This culture produced a yellowish-green pigment which was water soluble but seemed somewhat different from the green pigment commonly produced by pseudomonads.

Fig. 23. Pseudomonas sp. Strain 156. Note the rather delicate flagellum with short wavelength of about 1 μ and small amplitude.

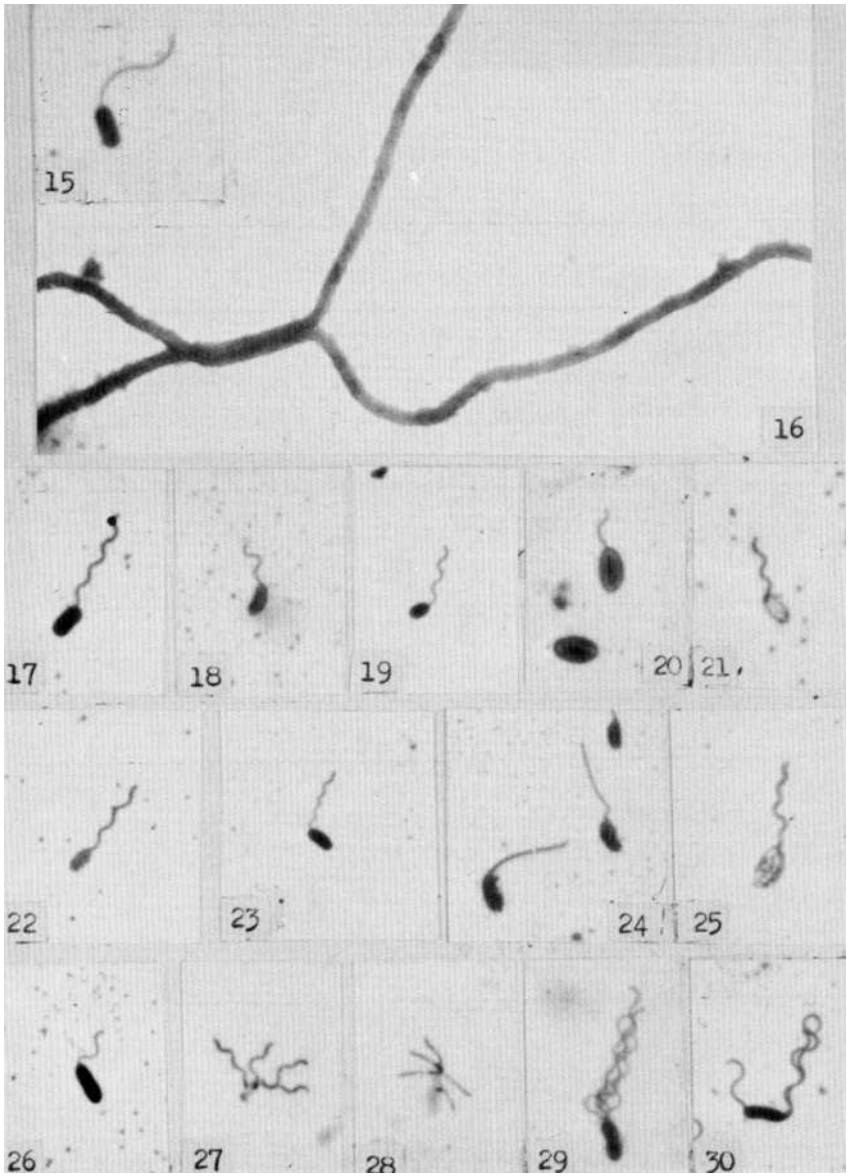
Figs. 24, 25. Pseudomonas sp. Strain 169. Figure 24 is the stain of a formalin fixed preparation and Figure 25 the stain of an unfixed preparation. Formalin sensitive flagella were somewhat rare among the distilled water bacteria.

Fig. 26. Pseudomonas sp. Strain 225. A halophobe oxidizing sucrose and maltose but not glucose.

Figs. 27, 28. Pseudomonas or Achromobacter? Strain 132. This organism was very tiny and the nature of the flagellation is not yet clear. The flagella are multiple with a strong polar tendency. Both pictures show one definitely lateral flagellum which should make the flagellation peritrichous and the organism a species of Achromobacter. The straight flagella shown in Fig. 28 are probably the result of the formalin fixation.

Fig. 29. Pseudomonas sp. Strain 93. A nice example of polar multitrichous flagellation. The flagella are unusually long.

Fig. 30. Pseudomonas sp. Strain 149. This is a typical polar multitrichous water pseudomonad. It produced melanin and reduced nitrate to nitrogen gas.



Plage II. Photomicrographs x 2,000. Leifson flagella stain.

Table 4. Some characteristics of the aggregating and selected

Strain	Pigment	Growth at 37°C	Growth in 1% NaCl	Gelatin	Nitrate	Catalase	Vitamin-free casamino acids	Salts glucose
127	- (melanin)	+++	-	-	-	-	-	-
6	orange	-	++	-	-	+	-	-
102	-	-	+++	-	-	+	+	-
297	yellow	-	-	-	-	++	++	++
10	"	++	-	-	-	+	+	++
234	"	+++	++	-	-	++	++	++
227	yellow	++	++	-	-	++	++	-
170	greenish-yellow	++	++	++	-	-	++	-
44	pink	-	-	-	-	-	-	-
71	"	+++	-	-	++	-	-	-
164	red	++	++	-	-	+++	+++	-
169	- (melanin)	++	+	-	++	-	++	-
149	- (melanin)	+++	+++	+	+ gas	+	+++	++
93	-	+++	+++	+	-	+	++	++
131	-	+	-	-	-	++	+++	-
253	green water sol.	++	+++	+	-	-	++	++
225	-	++	-	-	-	-	-	-
75	-	-	++	-	-	-	++	-
156	-	++	+++	-	-	+	-	-
84	-	-	+	-	++	-	+	-
201	green water sol.	+	++	+	++	-	++	-
132	-	-	++	-	-	-	++	++

polar flagellate types.

Acid aerobically, O-F medium								
Glucose	Sucrose	Maltose	d-Mannose	Xylose	Lactose	Flagellation W.L. in μ	Genus or group	Fig. No.
+	+	+	-	+	-	monotrichous 1.0	Caulobacter	
+	-	++	-	++	-	monotrichous 0.8		6-8
+	-	++	-	++	-	monotrichous 1.3		1-5
+	+	++	++	+	-	monotrichous 1.8	Aggregating	10, 11
+	+	+	+	+	-	monotrichous 1.6		9
+	+	+	+	+	+	monotrichous 1.8		15, 16
+	+	+	+	+	+	monotrichous 1.8	Xanthomonas	13
+	-	-	++	++	-	monotrichous 1.6		12
+	-	-	-	-	-	monotrichous 1.6		17
-	-	-	-	-	-	monotrichous 1.4		18
-	++	-	-	-	-	monotrichous 1.6		19
+	++	++	+	+	++	monotrichous 1.4		24, 25
+	-	++	++	+	-	multitrichous 2.3	Pseudomonas	30
++	-	++	++	-	-	multitrichous 1.4		29
++	-	++	++	+	-	multitrichous 2.0		
++	++	-	+	-	-	multitrichous 2.6		
-	+	+	-	-	-	monotrichous 2.0		26
?	-	-	-	-	-	monotrichous 2.2		20
-	-	-	-	-	-	monotrichous 1.0		23
-	-	-	-	-	-	monotrichous 1.7		21
-	-	-	-	-	-	monotrichous 2.1		
+	-	+	+	+	-	peritrichous? 1.3	?	27, 28