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MOTILE MARINE BACTERIA

I. TECHNIQUES, ECOLOGY, AND GENERAL CHARACTERISTICS

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ABSTRACT

LEIFSON, EINAR (Loyola University, Chicago, Ill.), B. J. COSENZA, R. MURCHELANO, AND R. C. CLEVERDON. Motile marine bacteria. I. Techniques, ecology, and general characteristics. J. Bacteriol. **87**:652-666. 1964.—Aerobic, heterotrophic bacteria were isolated from the waters of the Long Island Sound, Narragansett Bay, Atlantic Ocean, and from the intestine of a variety of marine animals found along the shore of the Long Island Sound. A total of about 600 cultures of motile bacteria were studied morphologically and physiologically, with special emphasis on flagellar characteristics. The great majority of the bacteria isolated from the water were polar flagellate, nonfermentative, nonpigmented, and gram-negative. Most of these were straight, capsulated rods, but a considerable number were curved like vibrios. Yellow-pigmented isolates were often nonmotile, and the motile forms were most frequently subpolar flagellate. Several rosette-forming bacteria, including *Caulobacter* species, were isolated. Two typical spirilla and one flagellated coccus were found. Peritrichous flagellate bacteria, both gram-positive and gram-negative, were rare except in bottom mud. The normal intestinal flora of marine animals, such as fish and shellfish, consisted of polar flagellate, fermentative, nonpigmented, gram-negative, straight rods. Curved forms, like vibrios, were less common. Polar multitrichous flagellate forms were not uncommon and included all the luminescent types isolated. A considerable proportion of the polar monotrichous flagellate rods swarmed over the surface of agar media. When grown on solid media, all of these showed mixed polar and lateral flagellation; in liquid media, mainly polar flagellation was found. The ecology and general taxonomy of marine bacteria are discussed.

To avoid misunderstanding, the following terms are defined: *psychrophiles*, bacteria which fail to grow at an incubation temperature of 37 C; *halophiles*, bacteria which fail to grow in salt-free media; *mixed flagellation*, essentially polar flagellation with development of lateral flagella of a different wavelength; *subpolar flagellation*, a single flagellum or a tuft of flagella originating laterally, usually close to the pole, and at right angles to the long axis of the soma.

MATERIALS AND METHODS

Collection of water samples. Water samples were taken from Noank Harbor (Long Island Sound) at various levels and from the bottom (about 10 m), from Narragansett Bay, and from the Atlantic Ocean (about 5,000-m depth). The Noank Harbor water was obtained with a modified Kemmerer water sampler, which had not been sterilized. The samples from the Narragansett Bay were obtained by the use of sterile bottles as described by ZoBell (1941). The Atlantic Ocean samples were obtained by the use of Nansen bottles, and some surface contamination cannot be excluded. The Noank Harbor water was plated a few hours after collection and also after 2 weeks of storage at laboratory temperature (15 to 25 C); this water was plated again after 6 months, after refrigeration for most of this time. The Narragansett Bay samples were plated after 2 days under refrigeration, and the Atlantic Ocean samples after 7 days, most of this time under refrigeration.

Flagellar staining. To 400 ml of selected samples of water was added 5% (v/v) of formalin (40% formaldehyde solution), and the bacteria were washed by centrifugation in preparation for flagellar staining by the method of Leifson (1960).

Plate counts and isolation of bacteria. For plate counts and for isolation, dilutions were made in sterile seawater, and 0.1-ml quantities were spread over the surface of natural seawater agar plates and salt-free nutrient agar plates. Duplicate plates were incubated at 20 and 37 C for 3 days, colonies were counted, and bacteria from

colonies which appeared different were transferred to broth. After incubation at 20 C, the cultures were examined for motility, and all nonmotile cultures were discarded.

Enrichments. The flagella stains of the bacteria concentrated directly from the water samples showed a considerable number of polar monotrichous vibrio forms which were quite rare in the cultures obtained from the plates. To obtain more cultures of these bacteria, one sample of the Noank Harbor water and one of the Narragansett Bay water were enriched by adding various combinations of nutrients. After several days of incubation at 20 C, the enrichment cultures were stained for flagella and plated on seawater nutrient agar. The following nutrients and combinations of nutrients were used (w/v): 0.1% potassium nitrate, 0.1% potassium nitrate plus 0.1% sodium acetate, 0.1% potassium nitrate plus 0.1% glucose, 0.1% ammonium chloride plus 0.1 glucose, 0.1% ammonium chloride plus 0.1% sodium acetate plus 0.001% sodium acrylate, 0.1% uric acid, 0.1% urea, 0.1% urea plus 0.1% sodium acetate, 0.1% urea plus 0.1% glucose, 0.05% ammonium chloride plus 0.05% urea plus 0.01% uric acid plus 0.05% potassium nitrate plus 0.05% sodium acetate plus 0.05% glucose plus 0.05% glucose plus 0.001% sodium acrylate.

Animal and plant samples. A variety of marine organisms, mainly animals, were collected from Noank Harbor, and the intestinal contents were streaked on the surface of artificial seawater nutrient agar plates. The plates were incubated at 20 C, with occasional duplicate plates incubated at 37 C. The plates were examined after 2 to 7 days, and all colonies which appeared different were transferred to broth. The nonmotile cultures were discarded as before. The sources of cultures were: sea weed (fluid in hollow stem), sea cucumber, egg case of *Urosalpinx*, crushed sea slug, barnacle, limpit, starfish, crab, mussel, sea clam, oyster, snail, and several kinds of fish.

Culture media. The culture broth and the plating agar were prepared with seawater taken from Noank Harbor and filtered through a 0.45-μ Millipore filter. To the water were added 0.2% Casitone (Difco) and 0.1% yeast extract; the pH was adjusted to 7.5; the mixture was then boiled, filtered through paper, and sterilized by autoclaving. This was the broth used for primary culture and for flagellar staining. For slants and plates, 1.5% agar was added to the broth. In studies of the isolated bacteria, artificial seawater was used in all media. Comparative studies showed that all the isolates grew equally well on media prepared with artificial seawater diluted with an equal quantity of distilled water, compared with media made with undiluted artificial seawater or with undiluted natural seawater. Since the acid-base buffer content of these media is low, it was advantageous to add additional buffer. Tris(hydroxymethyl)aminomethane (tris) buffer in 0.05% concentration proved to be satisfactory. The salt-free agar had the same composition as the seawater agar, but with distilled water in place of seawater.

Gelatin and nitrate. Tests for gelatin liquefaction and nitrate reduction were made using a single medium of the following composition: artificial seawater (full or half strength); Casitone (Difco), 0.2%; yeast extract, 0.1%; gelatin, 8.0%; sodium nitrate, 0.2%; and tris buffer, 0.05%. After heating to dissolve the gelatin, the pH was adjusted to 7.5 with HCl, and the medium was tubed and autoclaved. The stab-inoculated medium was incubated at 20 C up to 7 days, unless liquefaction was apparent sooner. The test for nitrite was made by adding two to four drops of reagent 1 (4 g of sulfanilic acid dissolved in 500 ml of 0.2 N acetic acid) followed by two to four drops of reagent 2 (2.5 g of α-naphthylamine acetate in 500 ml of 0.2 N acetic acid).

Reduction of nitrate with gas formation was apparent in the form of bubbles in the solid medium. In the few instances where the gelatin liquefaction was extremely rapid, gas formation was checked by using a liquid nitrate medium with an inverted vial to trap the gas.

Carbohydrates. The tests for carbohydrate metabolism were made with the MOF medium of Leifson (1963) and the following carbohydrates: glucose, sucrose, lactose, xylose, maltose, and mannitol. The incubation temperature was 20 C, and the tubes were discarded after 7 days.

Indole. The basic culture broth with 0.5% Casitone was satisfactory for the indole test with Kovac's reagent.

Catalase. Catalase was determined by addition to agar-slant cultures of commercial 40% hydrogen peroxide diluted 1:10 (v/v) with distilled water.

RESULTS

Temperature and osmotic relations. The plate counts of the various water samples at 20 and 37 C with seawater and salt-free agar media

showed the great majority of the bacteria to be psychrophilic and halophilic. A sample stored at room temperature (15 to 25 C) for 2 weeks showed a decrease in plate count to less than 1% of the original count, with seawater agar and incubation at 20 C. The plate count with seawater agar at 37 C was close to the original, as was the plate count with salt-free agar at 20 C. The psychrophilic bacteria in the water evidently died rapidly on storage at room temperature.

Morphological types in water. On direct flagellar staining, the most common type seen in all of the water samples was a small rod without flagella. Next in order was a small rod with a very short polar flagellum of variable curvature. Vibrio forms and short spiral forms were occasionally seen in the Noank Harbor water but not in the Noank Harbor mud or the water from the Atlantic Ocean. Occasionally, there were seen small rods with mixed flagellation, rods with several polar flagella, and rarely a type which appeared to be stalked. All of these were later isolated in pure culture. Yeasts, protozoa, and algae were seen in all of the samples except those from the Atlantic Ocean. The Narragansett Bay samples were too small, and the bacteria too few, for satisfactory flagellar staining.

TABLE 1. *Incidence of some morphological and physiological types of bacteria isolated from various marine environments*

Source	Total isolates	Motile	Polar flagellate*	Fermentative*
		%	%	%
Noank Harbor bottom mud	19	47	44	11
Noank Harbor water	95	39	97	8
Noank Harbor water stored 2 weeks	86	58	90	0
Noank Harbor water stored 6 months	20	95	95	0
Narragansett Bay water	53	75	98	10
Atlantic Ocean water	39	79	87	0
Water enrichments	47	98	100	7
Odd marine organisms†	25	88	100	5
Limpits	15	93	100	36
Snails	26	77	100	55
Starfish	9	78	100	71
Bivalves‡	134	85	98	38
Crabs	20	95	90	74
Fish	119	95	99	85

* Expressed as percentages of motile isolates.

† Includes sea cucumber, egg case of *Urosalpinx*, slug juice, barnacle.

‡ Includes clams, mussels, oysters.

After the water samples had been plated and the various colonies cultured, the number of cultures of vibrio-shaped organisms was very small and out of proportion to the number seen on direct staining. By plating the Noank Harbor water and the Narragansett Bay water on seawater agar containing 0.1% ammonium chloride, 0.1% sodium acetate, and 1.5% agar, three cultures of vibrio-shaped organisms were isolated. Eleven more organisms of this type were isolated by the enrichment techniques. Many vibrio-shaped organisms were seen on direct staining of the enrichment cultures, but only from the enrichments without glucose were these organisms isolated in pure culture. The best enrichments for this purpose were potassium nitrate, urea, and uric acid, with and without sodium acetate.

Motility of isolates. In Table 1 is recorded the incidence of motile and nonmotile cultures isolated from the various sources. In the Noank Harbor mud samples, the nonmotile and motile isolates numbered about the same. From the fresh Noank Harbor water, the motile isolates were only 39% of the total. When this water was stored for about 2 weeks, the proportion of motile isolates increased to 58%, and after 6 months of storage the proportion of motile isolates had increased to 95%. Storage of seawater evidently favors the survival of the motile bacteria over the nonmotile bacteria. The water samples from Narragansett Bay and from the Atlantic Ocean yielded a much greater proportion of motile cultures than the water from Noank Harbor (75 and 79%, respectively). Neither of these samples, however, was plated immediately after collection, and this may have increased the proportion of motile isolates. The proportion of motile isolates from the enrichments and from the various marine animals was definitely greater than from the water samples. Of the 119 cultures isolated from fish intestine, 95% were motile.

Flagellation of isolates. The motile isolates were stained for flagella, and Table 1 shows the incidence of polar flagellates, including subpolar and mixed flagellation, and nonpolar or peri-

trichous flagellates. From the Noank Harbor mud samples, the peritrichous types actually outnumbered the polar types, but from all the other samples the proportion of peritrichous types was very low. From fish intestine, the proportion of peritrichous types was less than 1%.

Carbohydrate metabolism. A striking difference in the bacterial flora of the water and the marine animal intestines is shown in Table 1. Of the motile isolates from the seawater samples, the proportion of fermenters averaged 4%. In contrast, the proportion of fermenters from fish intestine averaged 85%.

Summary of ecological characteristics. Motile gram-positive bacteria, including sporeformers, are rare in pure seawater and the intestine of marine animals such as finfish and shellfish. These bacteria are very prevalent in bottom mud. Motile cocci are very rare from any source. Typical spirilla are rare and probably closely associated with marine animal life. The bacterial flora of pure seawater is predominantly gram-negative, with a varying proportion of motile and nonmotile types. Of the motile types, 95% or more are polar flagellate (mainly monotrichous) and nonfermentative. The intestinal flora of marine animals such as fish and shellfish is predominantly gram-negative, polar flagellate, fermentative, and anaerogenic. Of the polar flagellate fermenters, the great majority are monotrichous or show mixed flagellation, while 5 to 10% are polar multitrichous or lophotrichous. Enterobacteriaceae are normally absent from pure seawater and from the intestine of marine animals such as fish and shellfish.

Peritrichous flagellate bacteria. These morphological types apparently are rare in seawater and the intestine of cold-blooded marine animals but quite common in bottom mud. In the latter, they are frequently gram-positive and sporeforming. Figures 1 and 2 show fairly typical large gram-positive peritrichous rods. Figure 3 shows a rather odd gram-negative type isolated from clam intestine. Figure 4 illustrates an organism which was isolated in four instances from the Noank Harbor water stored for 2 weeks in the laboratory. This was a small gram-negative rod with one or two very long flagella of unusually long wavelength. The four isolates were culturally and physiologically identical and had the characteristics of *Alcaligenes*. Growth in both liquid and agar media was barely visible. Figure 5 illustrates a species of *Achromobacter*.

A very odd organism (XW14) was isolated from the intestine of a small perch. The physiological reactions set it apart from the usual Enterobacteriaceae: glucose was fermented without gas formation, indole-positive, gelatin liquefied, nitrate reduced with gas formation, other carbohydrates negative. The soma of the organism was pleomorphic and branching forms were seen frequently (Fig. 7). The flagellation was peritrichous, with flagella of both normal and curly curvature (Fig. 6).

Flagellated cocci. From a clam intestine were isolated three similar cultures which produced a water-insoluble orange-yellow pigmentation. The organisms were gram-positive and actively motile, with somatic groupings of one, two, and four individuals but never cubes of eight. Each coccus usually had one or two flagella and a few individuals had up to four flagella. The flagellar wavelength was about 3 μ (Fig. 8–11). The physiological characteristics of strain XQ58 are shown in Table 2. Morphologically and physiologically, the organism belongs in the genus *Micrococcus*.

Typical spirilla. The large spirilla with the characteristic curved soma and lophotrichous flagella appeared to be rare in seawater, since direct staining of the water samples did not show any organisms of this type. From the intestine of a mussel was obtained a mixed culture of a large spirillum (Fig. 12) and a polar monotrichous rod. Several attempts to isolate the spirillum in pure culture were unsuccessful. From the intestine of a snail was isolated a pure culture of a large spirillum (Fig. 13). This organism grew readily in the seawater broth and agar media. All physiological tests used were negative. Another colony from the plate of the same snail intestine yielded a mixed culture of a spirillum and a polar monotrichous rod. This spirillum was obtained in pure culture. Throughout the study the isolation of mixed cultures was exceedingly rare, and the two instances of mixed spirilla and rods may not be purely a matter of chance.

Polar flagellate curved rods. Two physiological types of small curved rods with polar monotrichous flagella were isolated. One type was fermentative and associated mainly with marine animals, and the other type was nonfermentative and free-living in the water.

The nonfermentative type appears to be ubiquitous in seawater but rare or absent in marine animal intestines. They grew very slowly and

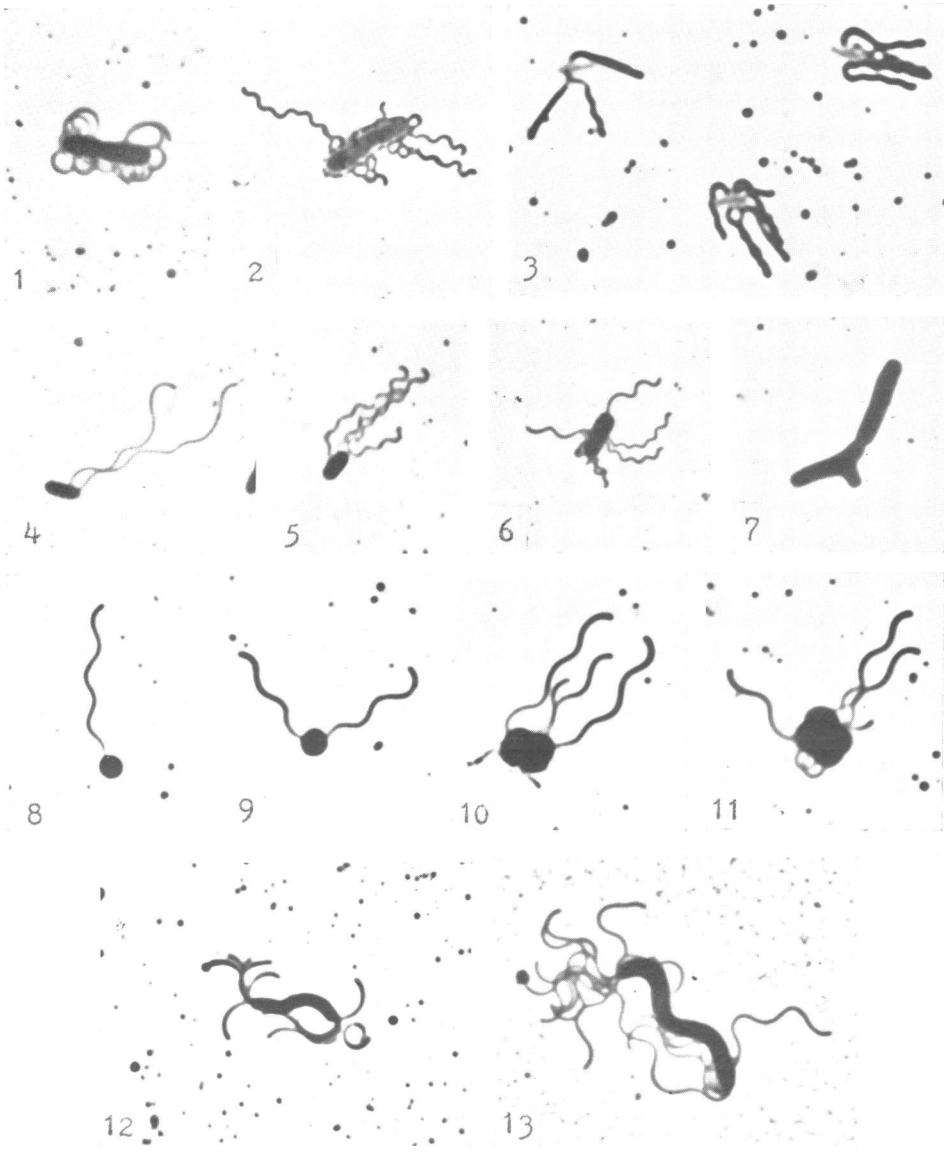

FIG. 1, 2. *Strains 3A5 and 24R3. Bacillus species isolated from bottom mud and from water of Noank Harbor. The coiled flagella of strain 3A5 are not unusual. (Leifson flagella stain and multiplication of ×2,000 used in all figures.)*

FIG. 3. *Strain XQ73. A gram-negative rod isolated from clam intestine. The peritrichous flagella are rather odd. A species of Achromobacter.*

FIG. 4. *Strain 1F28. A species of Alcaligenes with unusually long flagella.*

FIG. 5. *Strain XA11. An Achromobacter species from blowfish intestine.*

FIG. 6, 7. *Strain XW14. This was the only fermentative peritrichous flagellate organism isolated. It was very pleomorphic and branching forms were common.*

FIG. 8, 9, 10, 11. *Strain XQ58. A species of Micrococcus isolated from clam (quohog) intestine.*

FIG. 12, 13. *Strains YM14A and Snail I. Typical spirilla isolated from mussel and snail intestine, respectively.*

never abundantly in the media used. In broth, most of the cultures produced a barely visible turbidity. This may account for the fact that few were isolated by direct plating; most of the 20 cultures were obtained from the water enrichments. Some of the cultures oxidized glucose and other carbohydrates with definite acidity; the majority showed no effect on carbohydrates, and the only positive physiological test was nitrate (Table 2, strains YM8 and 1T4). The flagellation shows some interesting features which tend to relate these bacteria to the spirilla. The small individuals with a single curve invariably have one polar flagellum. Individuals with two curves frequently have two polar flagella, and those with many curves frequently have many polar flagella. The latter forms are not unlike small spirilla (Fig. 17 and 18). Figures 14, 15, and 16 are photomicrographs of organisms in water enrichments and were not isolated in pure culture. The somata of the organisms in Fig. 14 and 15 are like those of small spirilla, but the flagellation is not.

A total of 14 cultures of curved, fermentative, polar monotrichous organisms were isolated, 12 from marine animal intestines and 2 from water. These organisms are not always readily recognized as curved types. The capsule may completely obscure the somatic curvature. When these bacteria form filaments, the filaments have a helical shape and the somatic curvature is obvious (Fig. 20). In the organism illustrated in Fig. 19, the curvature of the soma is obvious both in the single and in the filamentous form. Physiologically, the group was very heterogeneous, with at least nine different types. Table 2 gives the physiological characteristics of the two major groups, represented by strains XA1 and 1C2.

Polar monotrichous flagellate straight rods. A straight rod-shaped bacterium with a predominantly single polar flagellum was the most common type of motile marine bacterium, both in the water and the marine animal intestines. In the water itself these bacteria accounted for 91% of the motile isolates and were mainly nonfermentative and nonpigmented. In the marine animal intestines, these bacteria accounted for 89% of the total isolates, with the larger proportion fermentative.

Many of these bacteria, both fermenters and nonfermenters, showed mixed flagellation, with a single polar flagellum of normal curvature and several lateral flagella of curly curvature. Although mixed flagellation has been observed with nonmarine bacteria of several kinds, it appears to be much more common among the marine bacteria. Mixed flagellation was observed only with the polar monotrichous straight rods, and never with the curved rods or the polar multitrichous rods.

Of the eight isolates which digested agar, all were polar monotrichous straight rods, with one showing mixed flagellation. Two of these cultures were carbohydrate fermenters and six were nonfermenters. The group was physiologically quite heterogeneous.

Some of the plates streaked with intestinal contents of marine animals showed swarming colonies. None of the plates prepared directly from water showed swarming colonies. Flagella stains of broth cultures from the swarming colonies showed only polar monotrichous rod-shaped bacteria. However, when agar-slant cultures were stained from distilled-water suspensions without formalin fixation or washing by centrifugation, the picture was entirely different: scattered over the slide were numerous, loose, curly flagella; some individuals with a normal polar flagellum and lateral curly flagella; and some with lateral curly flagella only (Fig. 35 and 36). Many of the agar-slant cultures which showed lateral curly flagella did not swarm. A study of temperature relationships showed a positive correlation between swarming and ability to grow at 37 C. Only carbohydrate fermenters swarmed.

Of the 185 polar flagellate, fermentative cultures isolated, 82% were polar monotrichous straight rods. Most of these were capsulated, 24% showed mixed flagellation, and 10% swarmed on agar to a greater or lesser extent. Physiologically, the group was quite heterogeneous and could be divided into 29 subgroups on the basis of nine physiological tests. Table 2 shows the characteristics of the five largest subgroups. The largest subgroup included almost one-half of the cultures (48%) and was fairly homogeneous; 95% of this subgroup were isolated from marine animal intestines and evidently comprise the most characteristic bacteria in the intestine of fish and shellfish. Some further statistics on this subgroup: 32% showed mixed flagellation, 24% swarmed on agar, 35% grew at 37 C, 77% were catalase-positive, 3% produced melanin, 3% produced a yellowish pigment, and one culture was agarolytic.

TABLE 2. *Characteristics of representative strains of the major groups of marine bacteria**

Representative strain	No. of similar strains found	Morphology	Water-insoluble pigment	Growth at 37 C	Gelatin liquefaction	Nitrate reduction	Indole	Utilization of						Remarks
								Glucose	Sucrose	Lactose	Xylose	Maltose	Mannitol	
XA4	73	Straight rods Monotrichous	–	+	+++	+++	+++	+++	+++	–	–	+++	+++	Fermentative. Mixed flagellation common. Major type found in marine animal intestines.
YSC7	10	Straight rods Monotrichous	–	+++	+++	+++	+++	+++	+++	+	–	+++	+++	
XE1	12	Straight rods Monotrichous	–	++	+++	+++	–	+++	+++	–	–	+++	+++	
1C3	13	Straight rods Monotrichous	–	–	+	+++	–	+++	+++	–	–	–	+++	
XV1	8	Straight rods Monotrichous	–	+++	–	+++	–	+++	+++	–	–	+++	–	
XA1	2	Curved rods Monotrichous	–	–	–	+++	–	+++	+++	–	–	+++	+++	Fermentative.
1C2	6	Curved rods Monotrichous	–	–	+++	+++	+++	+++	+++	–	–	+++	+++	
YSC10	13	Lophotrichous		+/–	–	+++	–	+++	+++	–	–	+++	+++	Includes all luminescent types. Fermentative.
YBC1	4	Polar multitrichous	–	++	–	+++	–	+++	–	–	–	–	–	Fermentative.
XA5	43	Straight rods Monotrichous	–	–	+++	–	–	++	++	–	–	+++	–	Nonfermentative. Some with mixed flagellation. Major type found in ocean water.
XB2	21	Straight rods Monotrichous	–	–	+++	–	–	++	++	++	++	++	++	
24R4	15	Straight rods Monotrichous	–	+	+++	++	–	++	++	–	–	++	–	
XF8	13	Straight rods Monotrichous	–	–	+++	–	–	++	++	++	–	++	++	
XP15	13	Straight rods Monotrichous	–	–	–	+++	–	–	–	–	–	–	–	
YM8	8	Curved rods Monotrichous	–	++	–	+++	–	–	–	–	–	–	–	Nonfermentative. Common in ocean water.
1T4	3	Curved rods Monotrichous	–	–	–	–	–	–	–	–	–	–	–	

XP35	4	Monotrichous	Yellow	+++	++	−	−	++	++	++	++	++	−	Xanthomonad types. Nonfermentative.
2F1	4	Subpolar monotrichous	Yellow	+++	−	−	−	+	−	−	−	+	−	Nonfermentative.
2F5	4	Aggregating monotrichous	Yellow	++	−	−	−	+	−	−	++	+	−	Aggregating or rosette-forming. Nonfermentative.
1F2	4	Aggregating multitrichous	−	−	−	−	−	+	−	−	+/−	−	+	
2F26	6	Stalked monotrichous	−	+++	+	−	−	++	−	−	−	−	−	*Caulobacter* types.
1F28	4	Peritrichous	−	−	−	−	−	−	−	−	−	−	−	
XQ58	3	Coccus peritrichous	Yellow-orange	+++	+++	−	−	++	−	−	−	++	++	*Micrococcus* type.

* A minus sign means a negative reaction; a plus sign, a weakly positive reaction; a +++ sign, a strongly positive reaction. None of the fermenters produced gas from the carbohydrates. Fermentative acid is indicated by **+**, oxidative acid by +.

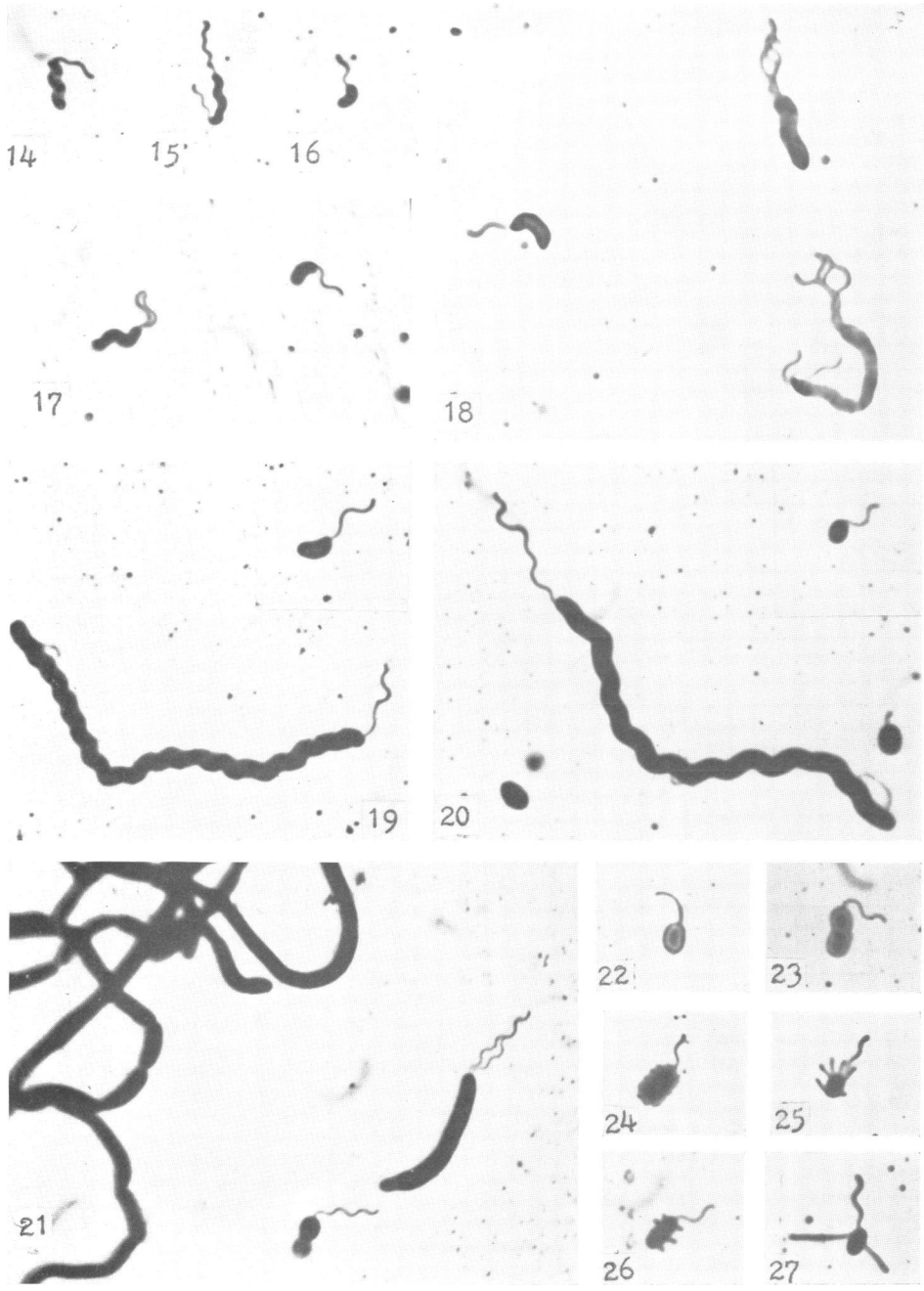

FIG. 14, 15, 16. *These small curved organisms were not isolated but were stained directly from seawater enrichment cultures.*

FIG. 17, 18. *Strains 10L6 and RI17. These illustrate a rather common phenomenon among the polar monotrichous, nonfermentative, curved rods. The single individuals have a single flagellum, and the longer spiral forms frequently have several flagella.*

FIG. 19, 20. *Strains XA1 and 1C2. In Fig. 19 (strain XA1) the single individual has a definite somatic curvature which explains the spiral nature of the filamentous form. In Fig. 20 (strain 1C2) the single individual has no apparent curvature and yet the filament is spiral. A capsule probably obscured the somatic curvature of the single individual.*

FIG. 21. *Strain 3L1. This organism was very filamentous. Note the two flagella on the long form and only one on the short form.*

FIG. 22, 23. *Strains 1G14 and 4K22. Marine bacteria are generally capsulated, but the flagella stain tends to stain the capsule and the soma is obscured. These figures show the small soma embedded in a dense capsule.*

FIG. 24, 25, 26. *Strain 1R6. The nature of the lateral projections has not been determined. Perhaps they are pili. In Fig. 24 the organism apparently is capsulated and the projections are partly covered.*

FIG. 27. *Strain 2F19. The two lateral projections may be straight flagella.*

Of the 538 polar flagellate cultures isolated, 353 (66%) were nonfermentative; of these, 247 (70%) were polar monotrichous straight rods. This was the largest group of bacteria isolated directly from the water. In the group were 27 cultures with mixed flagellation. The majority of the cultures appeared to be capsulated. Two cultures were agarolytic. Several of the cultures produced melanin, and in some cases the medium became almost black. Of 28 cultures producing some pigmentation, 17 contained a yellow-green water-soluble pigment; 9, a pink, red or reddish-brown pigment; 2, a purple pigment; and 6, a yellow water-insoluble pigment. About 50% were psychrophilic. Physiologically, the group was very heterogeneous and could be divided into 51 subgroups on the basis of nine physiological tests. Table 2 shows the characteristics of the five largest subgroups. In common with other nonfermenters, none of the cultures gave a positive test for indole. Four cultures produced gas from nitrate. The largest subgroup of 43 cultures was obtained from a variety of sources, both water and marine animal intestines, and was fairly homogeneous. In this subgroup were 11 cultures which showed mixed flagellation (Fig. 33 and 34). With eight of the cultures, the acid reaction with glucose was much weaker than that with sucrose or maltose.

Polar multitrichous flagellate rods. Of this type, 34 cultures were isolated. In the group were 23 cultures which fermented glucose (all of intestinal origin) and 11 cultures which either oxidized glucose or had no detectable effect. Of this latter group, seven originated from water and four from marine animal intestines.

The largest and most homogeneous subgroup consisted of straight or slightly curved rods with a typical tuft of polar flagella of long wavelength, which may be referred to as lophotrichous (Fig. 37, 38, 39). In this subgroup of 16 cultures were 5 cultures which were luminescent. No other luminescent cultures were isolated. It must be stated, however, that luminescent colonies were not specifically looked for, and the isolation of a luminescent organism was purely a matter of chance. All five of the luminescent cultures were identical morphologically, culturally, and physiologically, but were also identical with seven other cultures which did not show luminescence. The 16 cultures with the typical lophotrichous flagellation were psychrophilic and halophilic. The characteristics of a typical stain (YSC10) are shown in Table 2.

Among the seven other cultures in this group were four which were much alike. These four cultures originated from the intestine of a crab. The organisms were straight rods with polar multitrichous flagella of somewhat unusual appearance. Many of the organisms had one short and one long polar flagellum; others had several polar flagella which originated as one thick stem which distally separated into the distinct flagella (Fig. 40 and 41). These cultures also differed physiologically from the lophotrichous subgroup, as shown in Table 2 (strain YBC1). The remaining three cultures produced a yellow-brown pigment, and were physiologically heterogeneous.

There were 11 cultures in the nonfermentative group. Five of these were quite similar and formed a rather unique group of bacteria in that they aggregated into definite rosettelike patterns. The other six cultures in the group were physiologically heterogeneous. One of these cultures from blowfish intestine had the rather unusual morphology illustrated in Fig. 42.

Subpolar flagellate and yellow types. The subpolar (or lateral) flagellate and yellow-pigmented types are grouped together because many of the cultures with a yellow water-insoluble pigment often had flagella, usually one, which emerged at a right angle to the long axis of the soma. The origin of the flagellum varied from close to the pole to the center, and the distinction between subpolar and lateral is not definite. In this group were 37 cultures, all nonfermentative.

Of the 16 deep-yellow cultures, 1 showed peritrichous flagellation and might be classified in the genus *Flavobacterium*. Six of the cultures had single polar flagella and could readily fit into the genus *Xanthomonas* (Fig. 43). The physiological characteristics of strain XP35 are shown in Table 2. Two of the cultures showed somatic aggregation with rosette formation and single polar flagella.

The subpolar flagellate yellow cultures closely resembled each other morphologically and physiologically, and also closely resembled the nonpigmented subpolar flagellate types. The flagellation of these bacteria is illustrated in Fig. 44–47. All of these cultures, whether pigmented or not, gave a strong catalase reaction, but all other tests were either negative or weakly positive, as shown for strain 2F1 in Table 2. Except for one

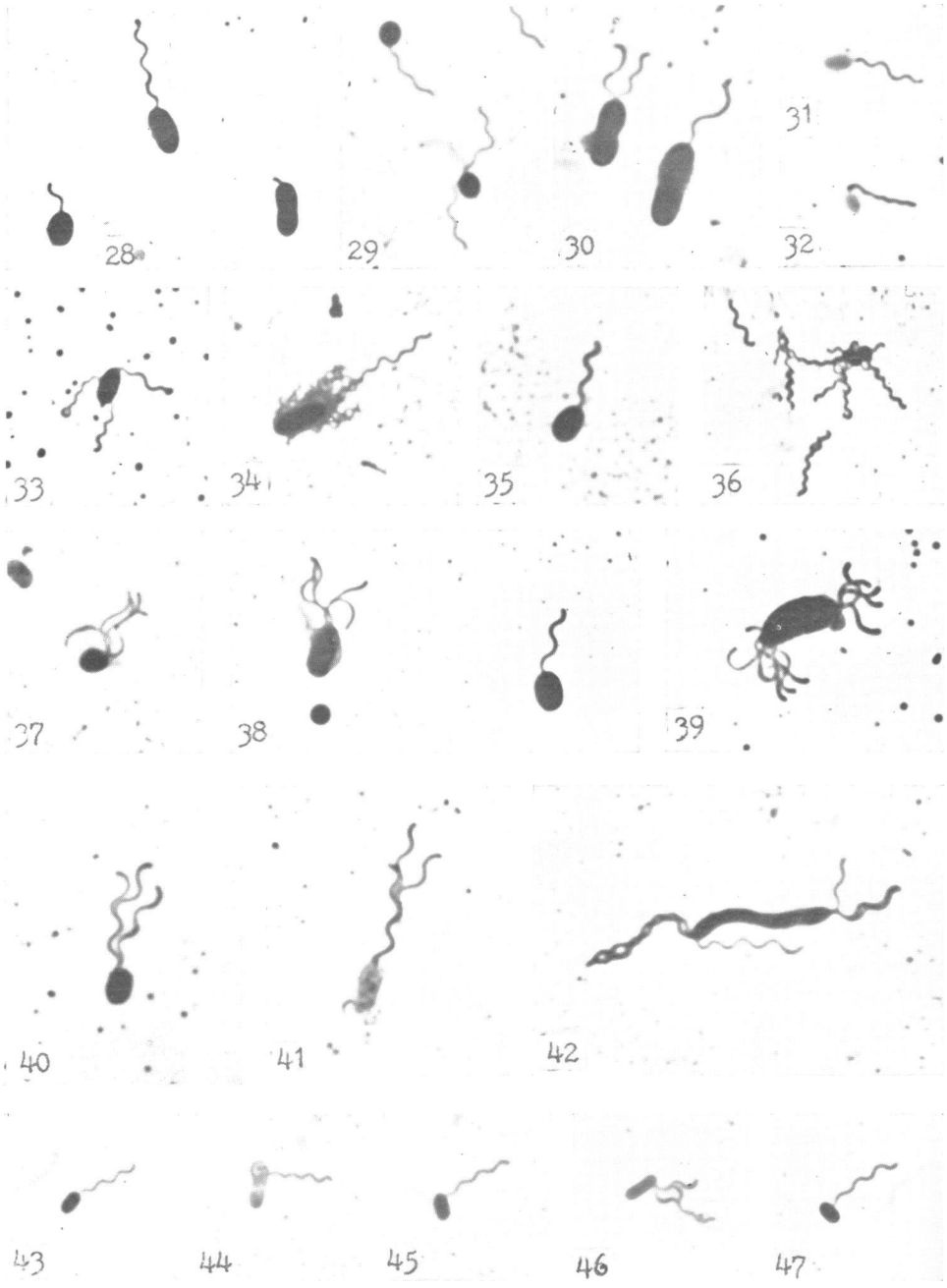

FIG. 28, 29, 30, 31, 32. *Strains 7L3, 3A6, 2N5, and XQ2. Figure 28 (strain 7L3) shows three stages in the growth of the flagellum. The mature organism is a typical example of polar monotrichous flagellate, capsulated, straight rod of marine origin. Figures 31 and 32 (strain XQ2) show very unusual polar flagella of distinctly different wavelengths.*

FIG. 33, 34. *Strains 2H7 and Y22. Examples of mixed flagellation.*

FIG. 35, 36. *Strains XD1. Figure 35 shows the typical appearance from broth cultures. Figure 36 shows*

culture isolated from clam intestine, all were isolated directly from seawater.

Aggregating or rosette-forming bacteria. Bacteria which form characteristic aggregates, usually in the form of rosettes, have been known for some time. The most striking, or most readily recognized, of these is *Caulobacter*. Three other polar monotrichous types of aggregating bacteria have been described by Leifson (1962), two isolated from distilled-water supplies and one from river water.

Six cultures recognizable as *Caulobacter* were isolated from the water of Noank Harbor. All were physiologically very much alike. Only one culture reduced nitrate to nitrite. Four cultures were positive for catalase; the two others were not. In Table 2 are shown the physiological characteristics of strain 2F26. The free-swimming individuals were small gram-negative rods, sometimes slightly curved, with a single polar flagellum of wavelength from 0.9 to 1.1 μ. In the seawater media the organisms showed considerable pleomorphism. Four of the cultures produced definite stalks and rosettes (Fig. 56–61), while two produced rosettes but no definite stalks.

Two cultures isolated from Noank Harbor water produced a yellow water-insoluble pigment and rosettes but no very definite stalks (Fig. 48–50). Short stalklike structures may be seen in Fig. 49 and 50. The free-swimming individuals, as well as those in the rosettes, were polar monotrichous flagellate with a flagellar wavelength of 1.4 to 1.7 μ, and thus distinctly different from the *Caulobacter* strains. Physiologically, they differed from the *Caulobacter* strains by oxidizing xylose and maltose, as well as glucose, with slight acidity (Table 2, strain 2F5).

Four cultures were isolated from Noank Harbor water, two directly and two from enrichments. They were nonpigmented; they formed characteristic rosettes but no definite stalks. The free-swimming individuals, as well as those in the rosettes, were gram-negative rods with polar multitrichous flagella (Fig. 51 and 52). A similar culture (XA9B) was isolated from the intestine of a small crab from Noank Harbor (Fig. 53–55). The two cultures isolated from the water enrichments were mesophilic. The other three cultures were psychrophilic and differed from the first two in one or more respects. Table 2 shows the general physiological characteristics.

Rosette formation by *Caulobacter vibrioides* is typically a matter of aggregation. The motile individuals attach themselves by the flagellated end to each other, to other bacteria, or to inanimate objects, and grow a stalk of variable length, depending on the chemical and physical environment. Preliminary study of strains 1F2, 2F5, and XA9B indicates that rosette formation by these organisms is not the result of aggregation of individual motile cells, but rather a growth phenomenon. It appears that these bacteria may multiply by budding as well as by binary fission.

DISCUSSION

Several characteristics are frequently encountered among marine bacteria which appear to be rare among nonmarine bacteria. One of the most striking of these characteristics is the formation of lateral curly flagella on polar monotrichous rods, both fermentative and nonfermentative. The fermentative rods with this mixed flagellation apparently constitute a considerable proportion of the normal flora of the fish intestine and the

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the appearance from slant cultures stained without washing by centrifugation. Scattered over the slide were loose curly flagella, and few organisms were apparent. Note the normal flagellum at upper left.

FIG. 37, 38, 39. *Strains 1G6, YP7, XG3. Examples of lophotrichous flagellation. Figure 38 of strain YP7 shows the typical flagellation of all the luminescent isolates. Individuals with a single polar flagellum were frequently seen among the lophotrichous individuals from the luminescent cultures.*

FIG. 40, 41. *Strains YBC1, YBC6. Examples of polar multitrichous flagellation. Several strains of this rather unusual type of organism were isolated from the intestine of a blue crab. With many individuals of the different strains, one flagellum was distinctly shorter than the other, as shown in Fig. 41. This is very unusual in polar multitrichous flagellate bacteria.*

FIG. 42. *Strain Blowfish A. This isolate from the intestine of a blowfish showed a long irregularly shaped soma and polar multitrichous flagella of rather short wavelength.*

FIG. 43, 44, 45, 46, 47. *Strains 1F23, 2F20, 3F2, 3F2, and 2F1. Examples of subpolar monotrichous and multitrichous flagellation. In some individuals in every culture the flagellum had a polar location as illustrated in Fig. 43. The subpolar multitrichous arrangement shown in Fig. 46 (strain 3F2) was seen in only one isolate and in only a few individuals.*

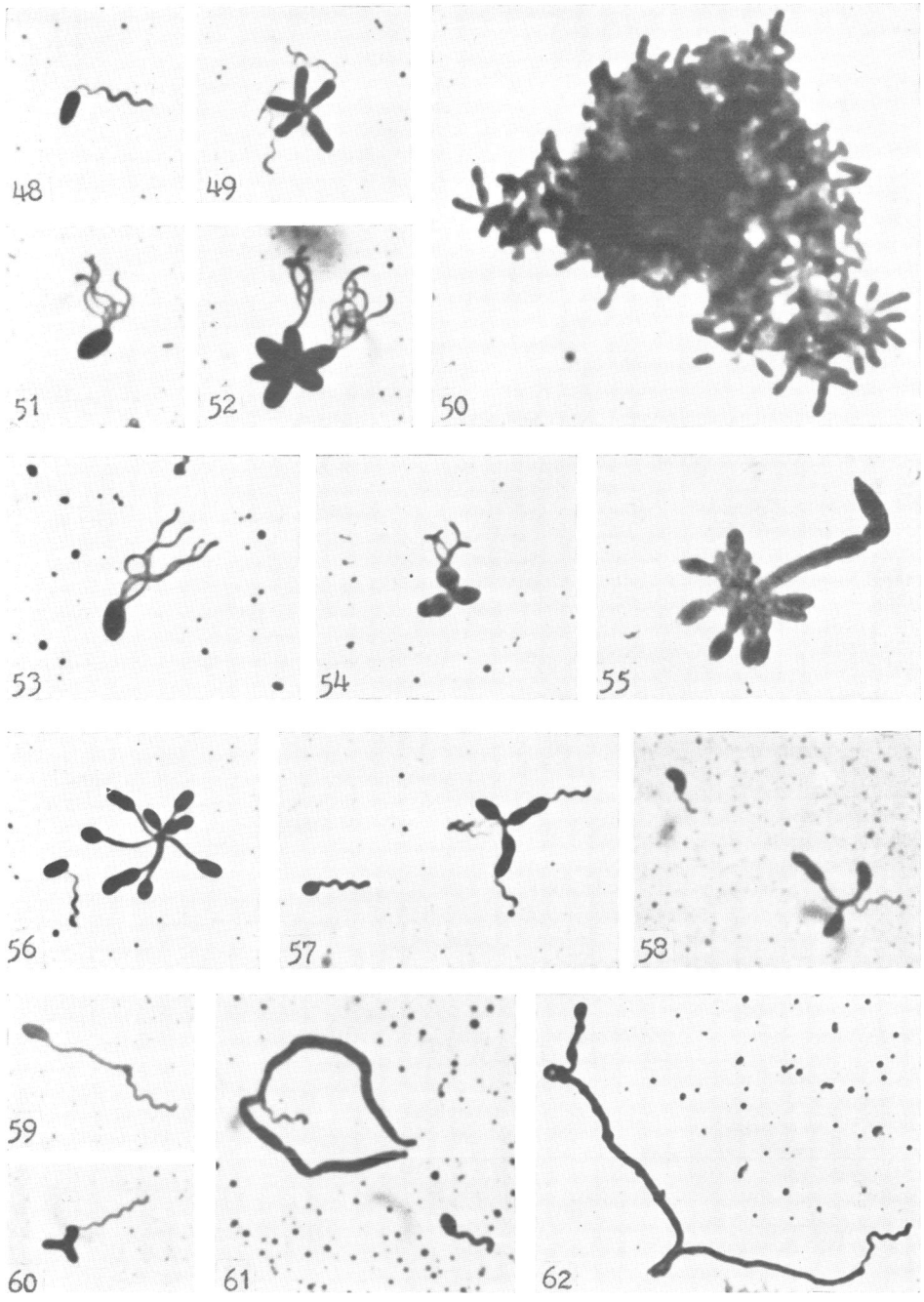

FIG. 48, 49, 50. *Strain 2F5. A yellow-pigmented aggregating type. The flagellation is polar monotrichous. Stalks are absent or rudimentary.*

FIG. 51, 52. *Strain 1F2. A beautifully flagellated organism with lophotrichous flagella, showing aggregation with rosette formation. Stalks were not apparent.*

FIG. 53, 54, 55. *Strain XA9B. The aggregation of this organism is less uniformly rosettelike than with strains 2F5 and 1F2. The organism multiplies by "budding" as well as by binary fission, and this may explain the aggregates. Long filaments frequently emanated from the aggregates (Fig. 55).*

FIG. 56, 57, 58, 59, 60, 61. *Strains 2F26, 2F26, 3F15, 3F25, 2F25. These are marine strains of Caulobacter. Stalk formation was quite variable among the different strains, and one strain did not produce any stalks at all. One strain was extremely pleomorphic (Fig. 61) with little differentiation between soma and stalk. Note the long stalk in Fig. 59 and absence of stalks in Fig. 60.*

FIG. 62. *Caulobacter seen in an enrichment culture of Noank Harbor water. The soma apparently is the small oval at left end, the rest being stalk with flagellum at end. Pleomorphism to this extent was seen only in the water itself.*

intestines of some other marine animals. Another striking feature is the almost complete absence of motile aerogenic bacteria from marine sources. Only a single organism was isolated which could be classified as *Escherichia*, *Aerobacter*, *Salmonella*, or *Proteus*. Only a single polar flagellate aerogenic culture was isolated.

The majority of the heterotrophic motile bacteria of seawater were nonpigmented polar monotrichous straight or slightly curved rods. The straight rods were typically capsulated and nonfermentative, and may be classified as species of the genus *Pseudomonas*. The great majority of these bacteria were psychrophilic and halophilic. Physiologically, the group was very heterogeneous and could be classified by conventional criteria into a considerable number of species. The small, slightly curved rods were typically noncapsulated, nonfermentative, psychrophilic, and halophilic. Many of these organisms grow very poorly on the usual media and show few positive physiological reactions. Their physiological characteristics and the tendency of the elongated or filamentous forms to develop multiple polar flagella would seem to relate them to the spirilla. However, it is our feeling at present that a new genus should be created for these bacteria. In some instances, differentiation from species of *Pseudomonas* may be a problem.

In contrast with the water flora, the majority of the motile bacteria from the marine animal intestines were nonpigmented, anaerogenic, fermentative, polar flagellate, straight rods. The majority of these were polar monotrichous flagellate, with a lesser proportion of polar multitrichous or lophotrichous flagellate rods. Both of these flagellar types may show a distinct somatic curvature. By the generally accepted criteria, an anaerogenic, fermentative, curved rod with a single polar flagellum is a species of *Vibrio*. The major difficulty here is recognition of the somatic curvature. It may be taxonomically advantageous to demote the significance of somatic curvature and to place all aerogenic and anaerogenic, fermentative, polar monotrichous and multitrichous rods in a single genus. The genus *Vibrio* seems unsuitable, with its connotation of somatic curvature. The genus *Aeromonas* also seems unsuitable, with its aerogenic connotation. As an alternative, the genus *Aeromonas* could be limited to include only the aerogenic types, and the anaerogenic straight and curved rods could be placed in a new genus, or a redefined genus *Vibrio*.

The taxonomic significance of mixed flagellation has yet to be evaluated. In this study, mixed flagellation was observed only with the polar monotrichous straight rods, both fermenters and nonfermenters. Both the fermentative and the nonfermentative groups were physiologically heterogeneous. Separate taxa for these morphological types do not seem justified at present.

The several agarolytic bacteria isolated had little in common except the ability to liquefy agar to a greater or lesser extent. There seems little or no justification for a separate genus for these bacteria. All the motile agar digesters isolated were polar monotrichous flagellate. Physiologically, the group was quite heterogeneous and included both fermenters and nonfermenters.

The five luminescent isolates were identical morphologically and physiologically, but were also identical with seven nonluminescent isolates. There seems little justification for a separate genus for the luminescent bacteria.

Motile organisms which produce a yellow water-insoluble pigment were not uncommon in the water flora but were rare in the marine animal intestines. A few of these isolates showed polar monotrichous flagellation, oxidative metabolism of carbohydrates, and may be classified as species of *Xanthomonas*. More frequently, the flagellation was subpolar or lateral monotrichous. The proper classification of these bacteria requires further study. Peritrichous flagellate bacteria which produce a yellow water-insoluble pigment and which show oxidative metabolism of carbohydrates, if any, are rare. Marine species of *Flavobacterium* apparently are mainly nonflagellate.

The rosette-forming bacteria, both stalked and not, may not be as uncommon as the data would indicate. The stalk-formers were much alike and could be classified in the genus *Caulobacter* as a distinct species. Those not forming stalks could be classified as two distinct species, but further classification is not justified at present.

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