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Isolation and Characterization of Filterable Marine Bacteria[1]

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ABSTRACT

ANDERSON, J. I. W. (Northeast Shellfish Sanitation Research Center, Narragansett, R.I.), AND W. P. HEFFERNAN. Isolation and characterization of filterable marine bacteria. J. Bacteriol **90**:1713–1718. 1965.—By a process of double filtration of seawater, first through a membrane filter with a pore diameter of 0.45 μ and then through a membrane filter with a pore diameter of 0.22 μ, it was possible to isolate on the surface of the latter membrane a group of marine organisms not usually encountered by conventional techniques of pour plates or one-stage filtration. Many of the isolates could not be identified, but the largest single group belonged to the genus *Spirillum*; other isolates were placed in the genera *Leucothrix*, *Flavobacterium*, *Cytophaga*, and *Vibrio*. A group of four organisms which was not identified was characterized by the formation of large, club-shaped cells, 20 to 30 μ long. Of the 25 strains studied in detail, 22 required seawater for growth and 8 retained their filterable property after cultivation. No filterable bacteria were isolated from terrestrial samples.

The occurrence of filterable bacteria in seawater was reported by Oppenheimer (1952), who found up to 12 viable cells per milliliter of seawater after passage through a membrane with a pore diameter of approximately 0.4 μ; with filtration procedures, L forms of bacteria have been isolated from marine molluscs (Brisou, 1960).

In our laboratory, bacteria were shown to be present in seawater after passage through membrane filters (Millipore Corp., Bedford, Mass.) with an average pore diameter of 0.45 μ, and the unusual morphology of these strains prompted an investigation into the nature of filterable marine bacteria.

MATERIALS AND METHODS

Collection of samples. Surface seawater was collected from Narragansett Bay in sterile glass bottles and filtered in the laboratory within 15 min. Samples from a salt pond, a fresh-water pond, a ditch, and an Artesian well were similarly collected and treated. A 10-g sample of garden soil was shaken in 100 ml of physiological saline, centrifuged at 1,700 × *g* for 10 min, and the supernatant fluid was removed for filtration.

Isolation media. For the isolation of filterable marine bacteria, a nutrient concentrate of peptone (Difco), 0.5 g, and yeast extract (Difco), 0.5 g, in 100 ml of distilled water was prepared, and 10-ml amounts were dispensed in 250-ml Erlenmeyer flasks before autoclaving. Agar plates were prepared from a medium containing: peptone, 1 g; yeast extract, 1 g; Noble agar (Difco), 15 g; artificial seawater (Lyman and Fleming, 1940), adjusted to a salinity of 25‰, 1 liter. For terrestrial samples, seawater was replaced by distilled water in the same medium.

Filtration procedure. An all-glass filtration apparatus (Millipore Corp.) was used routinely, assembled with 47-mm membrane filters with an average pore diameter of either 0.45 μ (HA) or 0.22 μ (GS). The units were autoclaved after assembly with the filter in place. Immediately before filtration, the assembly was aseptically placed over a flask containing the concentrated medium, and a seal was effected by use of a rubber collar. Samples were filtered by use of a main-line vacuum with a cotton plug trap to prevent back contamination.

From each water sample, 40 ml were removed and added to 10 ml of concentrated medium. A second 40-ml sample was passed through an HA filter directly into the concentrated medium. A third 40-ml sample was passed through the same HA filter into an empty, sterile, 250-ml Erlenmeyer flask, and this filtrate was immediately refiltered through a GS filter into the concentrated medium. At this stage the filtration apparatus was disassembled, and the GS filter was aseptically removed and placed, effluent side down, on the surface of the agar medium. Thus, from each sample, three 40-ml portions were inoculated into growth medium—one before filtration, one after

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filtration through 0.45-μ pores, and one after filtration through 0.22-μ pores. In addition, the GS membrane was placed on the surface of the agar medium. On one occasion, after filtration of the sample through the HA filter, the membrane was washed by drawing through an additional 40 ml of sterile artificial seawater. The washings were then treated like other HA filtrates. All samples were incubated aerobically at 20 C.

Control of filtration procedure. Since results of filtration experiments such as those described are subject to criticism on the grounds of contamination or membrane imperfections, careful controls were implemented. To minimize the possibility of chance aerial contamination, all work was conducted in a bacteriological hood. To check the integrity of each sterilized HA filter membrane, before filtration of a sample, a loopful of a young culture of *Staphylococcus aureus* or *Serratia marinorubra*, in 50 ml of nutrient medium, was filtered. To rule out the possibility of distortion of the filter by sea salts, the *S. marinorubra* culture was suspended in seawater medium prior to filtration. In all cases, this filtrate remained sterile after prolonged incubation.

Culture media. The medium for the cultivation and maintenance of all isolates had the same nutrient composition as the isolation medium, i.e., peptone, 1 g; yeast extract, 1 g; artificial seawater with a salinity of 25‰, 1 liter. This medium will be referred to as YEP. Noble agar was added when necessary to give 2.5 g per liter (SAYEP), or 15 g per liter (AYEP).

Isolation of filterable organisms. After 7 days of incubation, colonies growing on the surface of the GS membrane were counted with a dissecting microscope, and a random sample of colonies was picked for further examination and inoculated into tubes of SAYEP. All cultures were plated on AYEP and, where necessary, purified by restreaking. Stock cultures were maintained in SAYEP at 5 C.

Characterization of isolates. Initially, 90 colonies were selected from eight samples and these strains were examined microscopically by use of wet preparations under phase contrast. On the basis of this observation, 25 isolates were selected for detailed study. The characters examined and recorded for each of these strains were: Gram reaction; cell morphology, phase contrast; motility; flagellation; oxidase (Kovacs); catalase; utilization of glucose (oxidative or fermentative); gelatin, casein, and starch hydrolysis; pigmentation; requirement of oxygen for growth; salinity requirement; filterability after cultivation. The details of most of these tests have been described (Anderson, 1962), but on this occasion YEP was used as the basic medium throughout.

Anaerobic growth was checked in YEP, under hydrogen, in anaerobic jars (model AJ2, Torsion Balance Co., Clifton, N.J.). The salinity requirements were examined in SAYEP prepared with distilled water and artificial seawater (Lyman and Fleming, 1940) to give salinities of 0, 2‰, 4‰, etc., to 30‰. Organisms, after cultivation in YEP, were subjected to filtration through HA and PH (0.3-μ average pore diameter) membrane filters.

Staining methods. Flagella stains were prepared by the method of Blenden and Goldberg (1965). Lipid inclusions were revealed by use of Sudan Black B with safranine as a counterstain. Both wet and dry mounts were examined.

RESULTS

Filterable organisms were isolated from every marine sample and from no samples of terrestrial origin. The number of organisms filtered varied widely from sample to sample, from less than 1 per milliliter to more than 30 per milliliter (Table 1). Sample I, which gave more than 1,000 colonies after one filtration through the HA filter, yielded at least 400 more colonies when the membrane was washed with sterile seawater.

Microscopic observation of the HA-filtered and unfiltered water, after 5 days of incubation in YEP, showed widely different cell populations, pointing to the selective nature of the technique (Table 1).

From eight seawater samples (B through I), 90

TABLE 1. *Numbers and types of bacteria isolated from different environments, before and after filtration*

Sample	Colonies developing after filtration of 40-ml sample	Predominant flora in liquid culture	
		Before filtration	After filtration
Seawater			
B	61	Motile rods	Motile rods
C	16	Motile rods	Swollen cells, round bodies
D	>12*	Motile rods	Spirals
E	38	Motile rods	Spirals
F	44	Motile rods	Spirals
G	76	Motile rods	Spirals
H	276	Motile rods	Rods and spirals
I	>1,000†	Unrecorded	Unrecorded
Salt pond	19	Rods	Rods and spirals
Ditch			
A	0	Varied	0
B	0	Varied	0
Lake	0	Rods	0
Artesian well	0	Rods	0
Soil	0	Varied	0

* Spreading organism interfered with count.
† Uncountable, estimated more than 1,000.

colonies were selected at random. Of these, 52 (58%) were spirilla, 2 were colorless trichomes, and the remainder were rods of various dimensions, mostly exhibiting extreme pleomorphism. Of these isolates, 25 were selected to give a cross-section of the morphological types, and these organisms were examined in detail (Table 2). All 25 strains were gram-negative, 10 strains were motile, and 9 of these had a single polar flagellum, the other motile strain being peritrichously flagellated. On the basis of morphology and biochemical reactions, it was possible to group the isolates and assign some of them to a genus.

A group of four organisms (C-1, C-4, C-5, C-6) was characterized by the irregularity of their morphology, which ranged from filaments 40 μ

TABLE 2. *Characteristics of filterable marine bacteria**

Strain	Morphology	Gram reaction	Pigment	Anaerobic growth	Oxidase	Catalase	Utilization of glucose	Hydrolysis of			Minimal salinity required for growth	Filterability after cultivation	Genus
								Gelatin	Casein	Starch			
											‰		
B-6	Small slender rods	−	−	−	−	+	K	−	−	−	8	+	
C-1, C-4, C-6	Large rods, "clubs," spheres	−	−	−	+	+	F	−	−	−	6	−	
C-2	Small slender rods	−	Yellow	−	+	+(W)	F	+	+	+	4	−	*Flavobacterium*
C-5	Large rods, "clubs," spheres	−	−	−	+	+	F	−	−	−	6	+	
C-7	Large rods, "clubs," peritrichous flagella	−	−	−	−	+	F	−	−	−	6	−	
C-10	Cocco-bacilli	−	−	−	+	+	F	−	−	−	2	−	
C-11	Cocco-bacilli	−	Yellow	−	+	+	F	+	−	+(W)	10	−	*Flavobacterium*
D-5	Spirals, polar flagellum	−	−	−	+	+	F	−	−	−	12	+	*Spirillum*
D-6	Spirals, polar flagellum	−	−	−	−	−	F	−	+	−	6	+	*Spirillum*
E-3	Spirals, polar flagellum	−	−	−	+	+(W)	F	−	+	−	10	+	*Spirillum*
E-10	Spirals, polar flagellum	−	−	−	+	−	F	+	−	−	10	+	*Spirillum*
F-6	Spirals, polar flagellum	−	−	−	+	+	F	−	−	−	16	+	*Spirillum*
G-1, H-4	Spirals, polar flagellum	−	−	−	+	−	F	−	−	−	10	+	*Spirillum*
G-3	Curved cocco-bacilli, polar flagellum	−	−	−	+	+	F	+	−	+	10	−	*Vibrio*
I-1, I-4	Cocco-bacilli	−	−	−	+	+	F	−	−	−	0	−	
I-3	Rods	−	−	−	+	+	F	+	−	−	10	−	
I-9	Rods, polar flagellum	−	−	−	+	+	F	−	−	−	0	−	
I-11, I-14	Trichomes	−	−	−	+	+	F	+	−	−	4	−	*Leucothrix*
I-12	Rods	−	Yellow	−	+	+	F	+	+	+	2	−	*Cytophaga*
I-13	Rods	−	−	−	+	+	K	−	−	−	10	−	

* A minus sign means a negative reaction; K, no acid detected; F, fermentation without gas production; W, a weak reaction, barely detected.

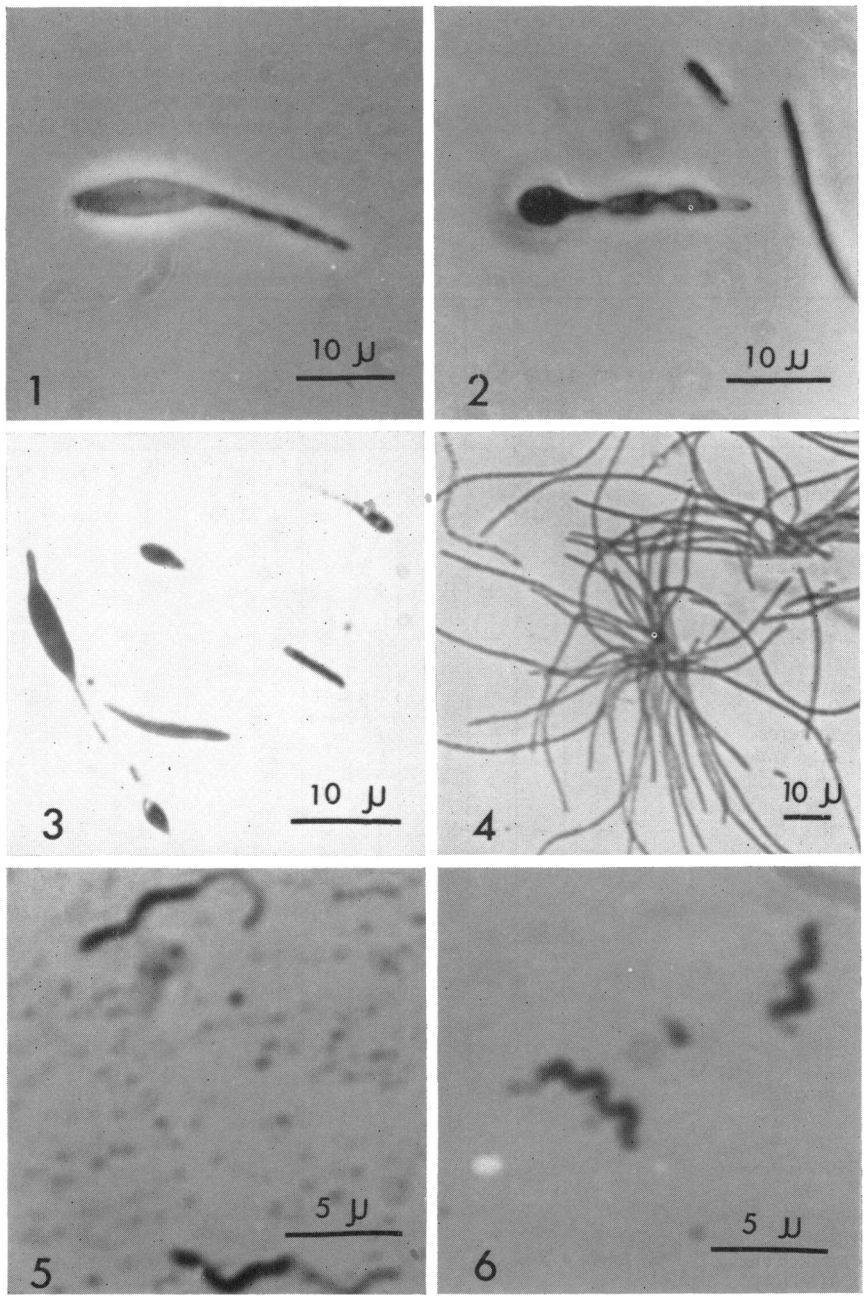

FIG. 1. *Typical club-shaped cell of strain C-1 from a 5-day culture in SAYEP at 20 C, shown as a wet preparation under phase contrast.*

FIG. 2. *Cells of strain C-1 from a 5-day culture in SAYEP at 20 C, showing lipid material demonstrated with Sudan Black B, counterstained with safranine and photographed as a wet mount under phase contrast.*

FIG. 3. *Same preparation as Fig. 2, heat-fixed and photographed under bright field.*

FIG. 4. *Wet mount under phase contrast showing the characteristic rosettes produced by strain I-11, cultured in SAYEP at 20 C for 3 days.*

FIG. 5 and 6. *Strains E-10 (Fig. 5) and F-6 (Fig. 6) showing spiral-shaped cells, each with a single polar flagellum. Taken from a 2-day culture in YEP at 20 C.*

long by 1.5 μ wide, to club-shaped cells with distinct swelling of one pole (Fig. 1), to spherical cells up to 5 μ in diameter. The clubs and spheres had large internal deposits of lipid material which could be demonstrated with Sudan Black B (Fig. 2 and 3). Isolate I-9, a large motile rod in seawater media, produced similar nonmotile irregular cell forms, including clubs and spheres with lipid inclusions, when cultivated in media prepared with distilled water. This led to the belief that the morphology observed in this group might be a cultural artifact, and on this basis these strains were examined under different conditions of salinity and temperature. Changes in temperature had no noticeable effect on morphology up to the maximal growth temperatures of 26 to 28 C, whereas reducing the salinity to 6‰ resulted in an increase in the length of filaments being produced. Isolate C-7 had a morphological appearance similar to this group, but was oxidase-negative and peritrichously flagellated, characters sufficiently important to establish a real dissimilarity.

Two isolates (I-11 and I-14) formed long colorless trichomes and characteristic rosettes (Fig. 4) and, from morphological characteristics, could be assigned to the genus *Leucothrix* (Harold and Stanier, 1955). This strain seems to be distinct from *L. mucor* (Harold and Stanier, 1955), though it may be closely related to *L. cohaerens* (Pringsheim, 1957). Further work will be necessary to define this strain accurately.

The largest single group of isolates had a distinct spiral morphology. All the spirilla showed one polar flagellum (Fig. 5 and 6); the cells were always irregular in size and shape and formed microcysts at some stage of their growth. Four of the seven strains examined lacked detectable catalase, and one of these was also oxidase-negative. All the others were strongly oxidase-positive. In the soft-agar medium, growth occurred as a surface pellicle and as a narrow band of growth about 1 cm below the surface. The cells were motile only in the presence of air.

The taxonomy of this group presented a problem because of the cells' distinct spiral morphology and single polar flagella. Williams and Rittenberg (1957), while discussing the taxonomy of the genus *Spirillum*, made reference to the confusion which surrounds the mode of flagellation of members of the genus. Under certain staining conditions most members of the genus appear to have polar tufts of flagella, but Williams and Rittenberg did not exclude organisms with single polar flagella, and stated that "distinct spiral form should be considered more important than the mode of flagellation in assigning an organism to the genus." In addition, there is a definite precedent for including strains with single polar flagella in the genus *Spirillum*, since *Bergey's Manual* describes *S. virginianum*, isolated from the mud on an oyster shell, as a strain with single polar flagella. Therefore, in consideration of the spiral morphology and microcyst formation, members of this group have been tentatively ascribed to the genus *Spirillum*.

Of the remaining isolates, two strains were placed in the genus *Flavobacterium*, one strain in the genus *Vibrio*, and one strain in the genus *Cytophaga*. The other six isolates were unidentified.

Twenty-two of the strains examined required some seawater for growth, even after prolonged cultivation in the laboratory. Eight strains retained their filterable property after cultivation, and two spirilla (D-6 and E-10) could be filtered through PH membranes. On no occasion was a viable particle detected after filtration through a GS membrane.

DISCUSSION

The use of membrane filters in marine microbiology has spread widely since first described by Oppenheimer (1952). Applications of the technique in the enumeration of marine bacteria were discussed by Jannasch and Jones (1959), and the membrane filter procedure was employed by Kriss (1963) during his investigations of the world oceans. Scholes and Shewan (1964) proclaimed, "The introduction of the membrane filter has undoubtedly been one of the major advances in the field [of marine microbiology]." From the results of this investigation and those presented by Oppenheimer (1952), it can be stated that membranes with a pore size of 0.45 μ cannot be relied upon to retain all cells, and quantitative estimates with the use of such membranes will have a tendency to produce low counts. Even membranes with a pore size of 0.3 μ allowed viable particles of two strains of spirilla to pass; thus, for complete retention of marine bacteria, a filter with a pore size of 0.22 μ is recommended. Since the bacterial counts in Narragansett Bay, as measured by plate counts, are in the range of 10^3 to 10^4 per milliliter, and filterable organisms usually numbered less than 30 per milliliter, any reduction in counts due to the loss of filterable particles would be insignificant. However, although membrane filtration with HA filters may suffer a small disadvantage in quantitative determinations, the technique of double filtration described here can have definite advantages in qualitative procedures, where certain species which are never, or rarely, isolated

on conventional spread plates, or by one-stage filtration, grow out as discrete colonies on the surface of the retaining GS membrane. For example, during this investigation spirilla were isolated routinely and a strain of *Leucothrix* was isolated once. Spirilla are not normally reported in the water of this area, though they may be obtained from seawater by a variety of enrichment and purification procedures (Williams and Rittenberg, 1957). During investigations of the bacterial flora of seawater of the northwest Atlantic, including Narragansett Bay, Leifson et al. (1964) failed to observe spirilla in the water, but did isolate spirilla from marine molluscs. In Narragansett Bay, members of the genus *Leucothrix* frequently can be identified microscopically as epiphytes associated with red algae (Brock, *personal communication*), though they are not isolated from seawater by conventional techniques. In nature the individual cells within the trichomes appear much larger than 0.45 μ, so it is surprising that this species was isolated after filtration. However, several species isolated during this study were not filterable after laboratory cultivation. The reason for this is unknown, although it could mean the existence of a life cycle, with the production of small bodies or reproductive elements only in the natural environment, or it could reflect a change in parent cell size in the rich environment of the culture vessel.

Although the isolates are not considered to be L forms as defined by Klieneberger-Nobel (1951), they do display certain similarities to stages of the L cycle. Round bodies and lateral swelling of cells were observed with the spirilla and *Vibrio* isolates. Lateral swelling of cells has been reported previously (Pease, 1956) to occur spontaneously in *S. undula* and after induction with penicillin in *S. serpens*, and the swellings were observed to liberate tiny cells. A filterable *Vibrio* was isolated from freshwater by Martin (1963), and Tuckett and Moore (1959), working with *Cellvibrio gilvus*, observed involution forms and cultured filterable particles. The club-shaped isolates regularly demonstrated pleomorphism, producing large swollen cells and spherical bodies, and the *Leucothrix* strain frequently produced large distorted cells at intervals along the length of the trichome. The relationship between the production of round bodies—or microcysts—and swollen irregular cells, and the filterable properties of these organisms, may not be entirely coincidental.

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