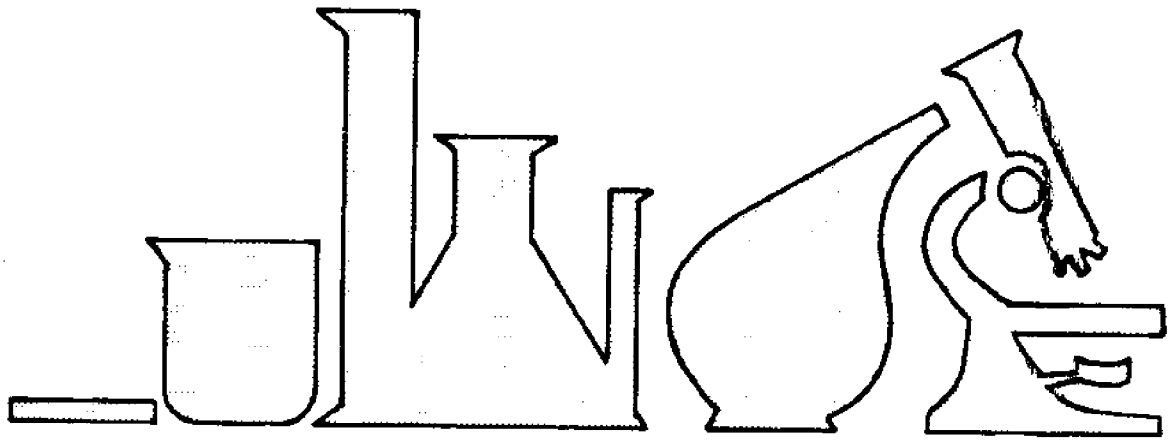
Study of the Ecology Of *Bdellovibrio bacteriovorus* In the Chesapeake Bay And Its Sub-estuaries

By William A. Falkler
And Henry N. Williams



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STUDY OF THE ECOLOGY

OF *BDELLOVIBRIO BACTERIOVORUS*

IN THE CHESAPEAKE BAY

AND ITS SUB-ESTUARIES

by
William A. Falkler
and
Henry N. Williams

Department of Microbiology
University of Maryland Dental School
Baltimore, Maryland

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Sea Grant Program
University of Maryland
1224 H. J. Patterson Hall
College Park, MD 20742

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PREFACE

The study of where and in what concentrations certain microbial populations occur is critical to monitoring the effects of pollution and the potential for disease in estuarine and coastal waters. These are waters used for recreation, industrial waste and sewage dumping, and fish food harvesting. Because microbial populations grow rapidly, responding almost simultaneously to changes in environmental conditions, they provide an early warning system for disturbances not yet detectable at the macro level. And because these populations exist in sympathetic or symbiotic communities, or in groups that respond similarly to given conditions, the presence of one easily detectable population may indicate the presence of other organisms.

In this context, Bdellovibrio bacteriovorus, a tiny, vibrio-shaped predator of gram-negative bacteria is of special interest. Many gram-negative bacteria are pathogenic, and those susceptible to bdellovibrios include Escherichia coli, Vibrio parahaemolyticus and Vibrio cholerae, all pathogens carried in water systems. Terrestrial strains of bdellovibrio have been isolated from soil, sewage, sludge and fresh water. Marine strains have been isolated from the Chesapeake Bay, other estuaries, and from coastal sea waters. Microbiologists William Falkler and Henry Williams have opened the study of this predator by investigating its occurrence and distribution in Maryland waters, its susceptible hosts, and the antigenic relationship of its marine and terrestrial strains.

Falkler and Williams found that marine bdellovibrios are isolated most readily from the mid and lower regions of the Bay where salinity is greater than one percent. The numbers increase in summer at higher water temperatures and decrease in winter to undetectable levels.

By direct culture of water samples, the investigators recovered greater numbers of bdellovibrios than any previous investigators, suggesting that this be the preferred technique for isolation. Testing for host susceptibility to bdellovibrio, they found that larger plaques formed in a natural sea water, polypeptone 20 and nutrient 20 media. Both findings should prove useful in future investigations. Bdellovibrios were found to have a wide range of susceptible gram-negative host bacteria.

Higher concentrations of bdellovibrios were found on submerged rope and oyster shell substrates than in the surrounding water. Other research has documented that vibrios also attach to surfaces. Higher concentrations of bdellovibrios on these surfaces may be related to the presence of vibrio hosts.

Marine strains of bdellovibrio did not share antigenic determinants with the terrestrial strain tested, but a Maryland marine strain did share determinants with a Mediterranean marine strain. Future investigations may refine the differences in the terrestrial and marine strains and their susceptible hosts.

As predators of gram-negative vibrios and coliforms, bdellovibrios may function to limit levels of certain populations of pathogenic

organisms in brackfish and coastal waters. Continued investigation may reveal a relationship between bdellovibrio numbers and their host pathogens. If so, bdellovibrio counts might be used to test for water quality.

Eventually, the study of aquatic microorganisms may lead to biological controls of water quality. Already a geneticist has invented and patented a microorganism that rapidly degrades spilled petroleum. Specific populations could be introduced to destroy or limit pathogenic populations as well.

--The Editors

INTRODUCTION

Bdellovibrio bacteriovorus is a predatory, intracellular parasite of many gram-negative bacteria. This endoparasite was first isolated by Stolp and Petzold (1962) while attempting to isolate bacteriophage specific for phytopathogenic pseudomonads from soil. These investigators noted that plaque was caused not by a bacterial virus, but by a small vibrio-shaped bacterium. Terrestrial and marine forms of this parasite have been described. The primary difference between the two types is their response when exposed to NaCl. The motility and attachment of the terrestrial form is inhibited by salt concentrations as low as 0.9% (Varon and Shilo, 1968; Shilo, 1966) while the marine organism requires salt during its growth cycle (Taylor et al., 1974).

The terrestrial bdellovibrios have been isolated from soil, sewage, sludge and fresh waters (Dias and Bhat, 1965; Klein and Casida, 1967; Staples and Fry, 1973; Stolp and Starr, 1963). Marine forms have been recovered from ocean or sea waters off the coast of Hawaii (Taylor et al., 1974), Japan (Miyamoto and Kuroda, 1975), in the Mediterranean (Shilo, 1966; Marbach et al., 1976) and bdellovibrio-like organisms have been observed off the coast of Massachusetts (Mitchell and Yankofsky, 1967). The marine form and its ecological relationship or significance to other forms of bacteria found in the same habitat have been investigated minimally. Yet this organism may be very important to the ecology of many gram-negative bacilli in the marine environment. In

areas where susceptible bacteria such as E. coli, other fecal organisms and V. parahaemolyticus and V. cholerae become a potential problem, removal by seeding with a predator such as Bdellovibrio bacteriovorus may offer a possible remedy, without the use of chemicals that may further upset the ecological niches.

V. parahaemolyticus is a pathogen found in marine food sources such as oysters and crabs. The first human infections by this vibrio in this country occurred in Maryland. Our laboratory tests showed that Bdellovibrio bacteriovorus isolated from the Chesapeake Bay is a predator of V. parahaemolyticus and a variety of other gram-negative bacteria indigenous to the Bay. The relatively low number of these pathogens may result from an ecological balance in which bdellovibrios may play an important part.

A study of the occurrence and number of terrestrial and marine forms of bdellovibrios in the Bay and its sub-estuaries should provide valuable ecologic information and could lead to a new index of the contamination of these waters.

This study initiates investigation of the following questions:

1. How widespread are marine bdellovibrios in the coastal waters of the eastern U.S., and specifically, what is their incidence in the Chesapeake Bay and its sub-estuaries?

2. What influences their presence? Are their occurrence, distribution, and numbers affected by seasonal changes, host populations, salinity, or other factors?

3. Can bdellovibrios be isolated from oysters, crabs, fish, and plankton? Are they involved in the biology of marine forms? And do marine animals and plants play a role in distributing bdellovibrios throughout the Bay?

4. What is the relationship of terrestrial and marine bdellovibrios to fresh and saline environments of the Bay?

5. Does the presence and number of these organisms per volume of sea water indicate the presence of coliform and other gram-negative bacteria associated with pollution?

6. Do bdellovibrios play a part in reducing or maintaining the numbers of coliforms and other susceptible bacteria, including V. parahaemolyticus, in areas where both occur? Several investigators have alluded to bdellovibrios as sources of population control for susceptible gram-negative bacteria with which they share their environment (Guelin et al., 1967; Mitchell and Morris, 1969).

7. Can addition of bdellovibrios to bodies of water be used to significantly reduce counts of undesirable bacteria in particular areas? This question is of special interest when one considers that V. parahaemolyticus, V. cholerae, and other pathogenic organisms

are known to occur in marine environments, have a history of causing disease, and are potentially damaging to the seafood industry in the state of Maryland.

8. What are the effects of major pollutants on the viability and infectivity of Bdellovibrio and on their role in conserving ecological systems?

These questions warrant the study of this important group of microorganisms. Their role in the ecology of the Bay may be a critical factor in the occurrence of coliform bacteria and human pathogens inhabiting Bay waters. As predators of coliforms and Vibrio parahaemolyticus, they may function to keep numbers of these gram-negative bacteria at a constant level.

The aims of the studies conducted were:

1. to determine the presence and seasonal distribution of freshwater and marine forms of bdellovibrios in areas of the Chesapeake Bay and its main freshwater tributaries;

2. to find their host bacteria in these waters;

3. to isolate these organisms from oyster shells, marine rope, and fish slime;

4. to test for elimination of gram-negative bacteria from water samples by the addition of Bdellovibrio; and

5. to research the antigenic relationship of marine and terrestrial bdellovibrio strains.

MATERIALS AND METHODS

Media. The composition of yeast extract agar (YEA), basal medium (BM) agar (Taylor et al., 1974), Marbach's M (PY10) agar (Marback et al., 1976), host sea water (HS) agar (Miyamoto and Kuroda, 1975), the modified polypeptone (PpX) agars and the nutrient broth (NBX) agars are listed in Table 1. Other media used for the culture of host bacteria or bacteria from water samples included polypeptone-salt (PpS) agar (Table 1), Trypticase soy agar (Baltimore Biological Laboratory (BBL), brain heart infusion agar (BBL), thiosulfate citrate bile salts (TCBS) agar (BBL), and sea water yeast extract (SWYE) agar (Kaneko and Colwell, 1978), the composition of which is also listed in Table 1.

All media used for the cultivation of bdellovibrios were used within one day of preparation or stored under vacuum to prevent dehydration. Natural sea water (filtered through 0.22μ Millipore filters), artificial sea water (ASW) diluent (Baumann et al., 1971) or distilled water was used for media preparation. The pH of all media was in the range of 7.5 to 8.6. In double-layer agar techniques, the bottom agar contained 1.5% agar (Difco) and the top agar 0.7% agar. Agar concentration used in media for total counts of bacteria and for the growth of organisms to serve as hosts was 1.5%.

Bdellovibrio strains. Marine strains of bdellovibrio isolated from various regions of the Chesapeake Bay, a strain isolated from coastal ocean water at Ocean City, Maryland designated OC1, two strains isolated on the Mediterranean coasts of Israel (BMS and BM11)

TABLE 1. Composition of media used to grow marine bdellovibrios, their potential hosts, and other organisms in the marine environment.

Yeast extract broth (YEB)

Tris (pH 7.5) - 50 mM
NH_4Cl - 190 mM
$K_2HPO_4 \cdot 3H\ 0$ - 0.33 mM
$FeSO_4 \cdot 7H_2O$ - 0.1 mM
Glycerol - 0.2%
K succinate - 0.2%
K acetate - 0.2%
Yeast extract - 5 g
Artificial sea water (ASW) -1000 ml

Basal medium (BM)

YEB without yeast extract

Nutrient broth (NB)

Nutrient broth - 8 g
Sea water - 1000 ml

Modification of NB (NBx)

Nutrient broth 10 (NB10)
Nutrient broth - 0.8 g
Sea water - 1000 ml
Nutrient broth 20 (NB 20 (NB20)
Nutrient broth - 0.4 g
Sea Water - 1000 ml

Sea water yeast extract (SWYE) agar

Proteose peptone - 1%
Yeast extract - 0.3%
Salts solution -1000 ml

Composition

NaCl - 2.4%
$MgSO_4 \cdot 7H_2O$ - 0.7%
KCl - 0.07%
$MgCl_2 \cdot 6H_2O$ - 0.53%
pH 7.6±0.2

Modified polypeptone-salt (Pps) agar

Polypeptone - 20 g
NaCl - 30 g
Agar - 15 g
Distilled water - 1000 ml

Modifications of Pps (PpX)

Polypeptone 2 (Pp2)
Polypeptone - 10 g
Sea water - 1000 ml
Polypeptone 10 (Pp10)
Polypeptone - 2 g
Sea water - 1000 ml
Polypeptone 20 (Pp20)
Polypeptone - 1 g
Sea water - 1000 ml

Host sea water agar (HS)

Host cells - 5 ml of $1x10^{10}$/ml
Sea water - 1000 ml

Marbach's M(PY/10)

Peptone - 0.05%
Yeast extract - 0.03%
Basal salt solution - 1 ℓ
NaCl - 28.15 g
$MgSO_4 \cdot 7H_2O$ - 6.92 g
$MgCl_2 \cdot 7H_2O$ - 5.51 g
$CaCl_2 \cdot H_2O$ - 1.45 g
KCl - 0.67 g

Artificial sea water (ASW) pH 7.4

NaCl - 0.4 m
$MgSO_4 \cdot 7H_2O$ - 0.1 m
KCl - 0.02 m
$CaCl_2 \cdot 2H_2O$ - 0.02 m
Distilled H_2O - 1 ℓ

obtained from Dr. M. Shilo, and a terrestrial strain Bd 110 (ATCC 15360) were utilized in these studies.

Suspensions of the organisms were prepared by innoculating the bdellovibrio strains into HS medium which contained appropriate gram-negative bacteria (10^8 - 10^9/ml) as host organisms. The dual cultures were placed on a gyratory shaker at room temperature or in a gyratory or reciprocal water bath shaker at 25° C and incubated until lysis of the host was apparent by the clearing of the culture fluid. After observation of bdellovibrios by phase microscopy, the culture fluid was centrifuged at 10,000 x g for 10 min and the resulting supernatant fluid decanted into a sterile centrifuge tube and centrifuged at 18,000 x g for 30 min. The supernatant fluid was decanted and the pellet resuspended in two to three ml of sterile diluent. The number of bdellovibrios and host organisms in the resuspension were quantified by viable plate counts using serial dilutions.

Bacterial strains as hosts. Organisms used as hosts or tested for their ability to serve as hosts for bdellovibrios and their sources are listed in Table 2. The organisms were grown on PpS agar at 25° C, except for the nonmarine strains which were grown in TSA agar at 30° C. After a 24- to 48-h incubation period, the hosts were harvested from the plates by flooding the plates with 10 ml of sterile sea water and suspending the host colonies in the sea water with glass spreaders. The optical density (OD) at 660 nm of the cell suspensions was measured and the viable number or organisms determined with

TABLE 2. Microorganisms tested or used as host bacteria.

Strain	Source
Vibrio species GP235g	Dr. Rita Colwell Department of Microbiology University of Maryland College Park, Maryland
Vibrio 1-JK presumptive	
Vibrio 2-JK presumptive	
Vibrio anquillarum ATCC 17802	
Vibrio marinus	
Beneckea nereida op	
Beneckea nereida tr	
Vibrio parahaemolyticus FC 1011	
Vibrio parahaemolyticus ATCC 17802	
Vibrio parahaemolyticus M5241	
Vibrio parahaemolyticus P-5	Dr. David Johnson Veteran's Administration Hospital Baltimore, Maryland
Vibrio parahaemolyticus P-6	
Escherichia coli ML 35	Dr. Robert Nauman Microbiology Department University of Maryland Baltimore Campus Baltimore, Maryland
Enterobacter cloacae ATCC 15351	American Type Culture Collection Rockville, Maryland
Pseudomonas LP-101	Dr. Moshe Shilo Department of Microbiological Chemistry Hadassah Medical School The Hebrew University Jerusalem, Israel

serial dilutions and the spread-plate method with PpS agar or TSA agar.

Diluents. In all experiments, serial dilutions were made in sterile water or with ASW (Baumann et al., 1971). The sea water used in the preparation of media or as diluent was collected off the beach at Ocean City, Maryland. The water was always collected at the same site (47th Street) in approximately three feet of water. Ten gallons of water were collected at a time, which allowed comparative experiments using the same batch of sea water. Prior to use, the water was filtered through 0.22μ disposable filters (Millipore) or through 0.2μ filters (Nalgene) and autoclaved.

Culture methods for bdellovibrio strains. Bdellovibrios were cultured on agar media using the double-layer agar technique (Stolp and Starr, 1963). Two-tenths ml of the host suspension (10^8 - 3 x 10^9 organisms/ml) was inoculated into three ml of melted top agar (48° to 50° C) in 13 x 100 mm tubes. One-tenth ml of the bdellovibrio suspension was then added, and the contents mixed and overlaid onto bottom agar plates. In some cases 0.1 ml of the bdellovibrio suspension was spread-plated onto the solidified surface of previously poured top agar plates containing host bacteria. Plates were incubated at 25° C for a minimum of 48 h and for as long as 10 days. Plaques were marked as they appeared, counted, and their diameters measured at daily intervals. Plates were usually examined for several days after initial counting in order to detect any new plaques which developed.

In liquid culture, bdellovibrios were grown in HS, Pp20, YEB and NB20 broths. The HS broth was used routinely to maintain bdellovibrios. For growth studies in liquid media, two ml of the bdellovibrio strains (1 x 10^7 cells/ml) and host bacteria suspensions (3 x 10^9 cells/ml) were added to 20 ml of each of the four media in 250 ml side-arm flasks. Host control flasks containing host cells without bdellovibrios were used. A set of uninoculated media flasks served as blanks in adjusting the spectrophotometer (Beckman Spectrophotometer 20). The side-arm flasks allowed the OD to be read quickly and with greater facility during the incubation periods.

Quantification of bdellovibrios and host cells. Dual cultures consisting of host cells and marine bdellovibrios required the use of two different media for the quantification of the respective bacterial species present. Appropriate serial dilutions made with sea water or ASW were cultured via spread or pour plates onto Pp20 double-layered agar for bdellovibrio counts (pfu) and onto PpS agar using the spread-plate technique for host cell colony counts. Plates were incubated at 25° C. The number of colonies on the PpS agar plates were counted within 48 to 72 h. Bdellovibrio plaques were usually counted later to allow ample time for development.

Host susceptibility. The susceptibility of 10 strains of marine bacteria and 2 nonmarine strains to lysis by strain OC1 was determined on five different media. YEA and M(PY/10) media were prepared with ASW and HS; Pp20 and NB20 media were prepared with natural sea water. The bacteria species tested were: Vibrio strains 1

and 2, V. anquillarum, V. parahaemolyticus strains P-5, P-6, FC 1101, ATCC 17802, B. nereida op, Vibrio strain GP234g, V. marinus, E. coli ML35 and A. cloaceae.

The procedure used to determine the susceptibility of the host organisms was the spot test method (Stolp and Starr, 1963) which was performed as follows: a single drop (approximately 0.4 ml) of the bdellovibrio suspension (5 x 10^6 pfu/ml) was placed onto a top agar surface which had been previously inoculated with appropriate host cells the day before. This was carried out with each host on the five different media. The plates were incubated at 25° C and were examined daily for a period of 10 days. A zone of lysis was recorded as positive, and negative results (a lack of zone of clearing) were not recorded until after the 10-day period.

Sampling stations. Water samples were obtained from the beach at Ocean City, Maryland and from the following Chespeake Bay stations listed in order from north to south: Jones Falls, Colgate Creek, Tolchester Beach, Chester River, Cook's Point, Patuxent River, Annamessex River, Smith Point at the Potomac River, the Rappahannock River, York River and Cape Henry in the mouth of the Chesapeake Bay. In the James River, samples were collected at Little Creek, Newport News and Sloop Point.

Collection and quantitative culturing of water for marine bdellovibrios and other bacteria. Water samples were collected aseptically in sterile containers. The "Niskin" sterile water sampler (General Oceanic, Inc., Miami, Florida) was used to collect top and

bottom water samples when samples were collected from aboard the research vessel (R/V) Ridgely Warfield. Approximately 50 ml of the collected samples were immediately transferred into sterile milk dilution bottles from the Niskin bags. Samples collected aboard the R/V, at Solomons Island, and some of the samples from Ocean City were cultured immediately upon collection. Other samples from Ocean City were transported to the laboratory and cultured approximately 4 h after collection. Samples from the Annamessex River were stored at 4° C overnight and transported to the laboratory for culturing the following day.

Samples were cultured in the following aliquots: 10 ml, 5 ml, 1 ml or 0.1 ml. The 10 ml and 5 ml samples were placed into 20 x 150 mm screwcap tubes containing an equivalent amount of Pp20 melted top agar (48° to 50° C). The agar and nutrient composition of this top agar was made at a concentration which allowed for the dilution effect of the added sample. Two ml of a suspension of V. parahaemolyticus P-5 (approximately 1 x 10^{9} organisms/ml) were added to the tubes of top agar. The tubes were mixed by inversion and the contents of the tubes overlaid onto Pp10 bottom agar in 150 x 25 mm petri dishes.

The 1 ml water sample to be tested was added to a 3 ml top agar tube following inoculation with 0.2 ml of the host suspension. The top agar tubes were mixed and overlaid onto the 100 x 15 mm bottom agar plates. The 0.1 ml sample was spread-plated onto the surface of hardened top agar containing host organisms. The 1 ml sample and the 0.1 ml sample were not

tested in duplicate since few pfu were expected on these plates. These were done in conjunction with duplicate plates of larger samples.

All plates, after hardening of the top agar, were inverted and placed in the incubator at 25° C as soon as possible. All plates were examined daily for plaques on the Pp20 medium for at least 7 to 10 days. Plaques without center colonies were marked as they appeared. Plaques were ascertained to be those of bdellovibrios based on size increases after their initial appearance and based on phase and/or electron microscopic observations of representative plaques.

Colony counts of bacteria on the SWYE and TCBS media were done after three days of incubation, unless overgrowth became apparent. In such cases, the plates were counted immediately or refrigerated until they could be counted.

Water samples collected during the winter months were too cold to add directly to top agar as the agar would harden immediately. In such instances, the water sample was warmed to room temperature prior to inoculating into the top agar. Alternatively, 1/2 ml of sample was spread-plated onto hardened top agar with host in 150 x 25 mm petri dishes.

Collection and quantification of sediment material. Sediment was collected with a grab-sampler. One gram of sediment was placed into a sterile 50 ml conical tube. Twenty ml of sterile sea water were added to the sediment and mixed by vortexing. The tube was allowed to remain stationary in an upright position until

the water became clear. Five ml of the supernatant fluid were drawn off and added to 5 ml of melted Pp20 top agar containing host cells. Cultural methods from this point were the same as for culturing water samples.

Collection of data at sampling stations. Physical and chemical measurements of the water taken at sites where samples were collected included, when possible: water temperature, conductivity, depth, dissolved oxygen content and transparency. The transparency was measured using a Secchi disc (Wildco Co., Saginaw, Michigan). The salinity was calculated from the temperature and conductivity measurements. The dissolved oxygen content was determined by the Winkler titration method. The temperature was obtained with a reversing thermometer.

RESULTS

Host susceptibility. The susceptibility of the 10 gram-negative marine bacilli listed in Table 3 to strain OC1 was determined using the spot test with five media. Two nonmarine strains were also tested with the Pp20 medium and strain OC1. A single drop (0.4 ml) of the bdellovibrio suspension was dropped onto the surface of top agar, the top agar being inoculated 24 h before with the host suspension. A positive test was observed as an area of clearing (Figure 1) and usually appeared after 48 h incubation; however, plates were observed for a period of 10 days. Strain OC1 formed an area of clearing when inoculated with 11 of the 12 host bacteria (Table 3) on Pp20 medium. Included were two nonmarine strains, Escherichia coli (ML35) and Enterobactor cloacae. The M (PY/10) medium demonstrated clearing with only 3 of the 10 host bacteria. The HS and NB20 media allowed OC1 lysis with 9 of 10 host strains, whereas the use of YEA resulted in the lysis of only 7 of the 10 hosts. Strain OC1 did not lyse P-6 on any of the media utilized. Addition of drops of autoclaved ocean water as a control resulted in no clearing of any of the host cells on any of the media used.

Seasonal distribution of Marine Bdellovibrios. Ocean and Chesapeake Bay surface water samples were cultured for marine bdellovibrios using a quantitative direct culturing technique. Water samples were taken from Ocean City (Table 4), Solomons Island and the Annamessex River (Table 5) over an extended period of time. Counts are expressed as the number of pfu

TABLE 3. Use of the spot test for determining host susceptibility with various bacteria and media.

Cultures	Pp20	HS	YEA	NB20	M(PY/10)
Vibrio 1	+[b]	+	-[c]	+	-
Vibrio 2	+	+	+	+	-
V. Anquillarum	+	+	+	+	-
V. parahaemolyticus P-5	+	+	+	+	-
V. parahaemolyticus P-6	-	-	-	-	-
B. nereida OP	+	+	+	+	-
V. parahaemolyticus ATCC 17802	+	+	+	+	+
Vibrio GP	+	+	+	+	-
V. parahaemolyticus FC 1101	+	+	+	+	+
Vibrio marinus	+	+	-	+	+
E. coli ML 35[a]	+				
A. cloaceae	+				

[a] Tested on Pp20 medium only.

[b] Zone of lysis detected.

[c] No zone of lysis detected within 10 days of incubation.

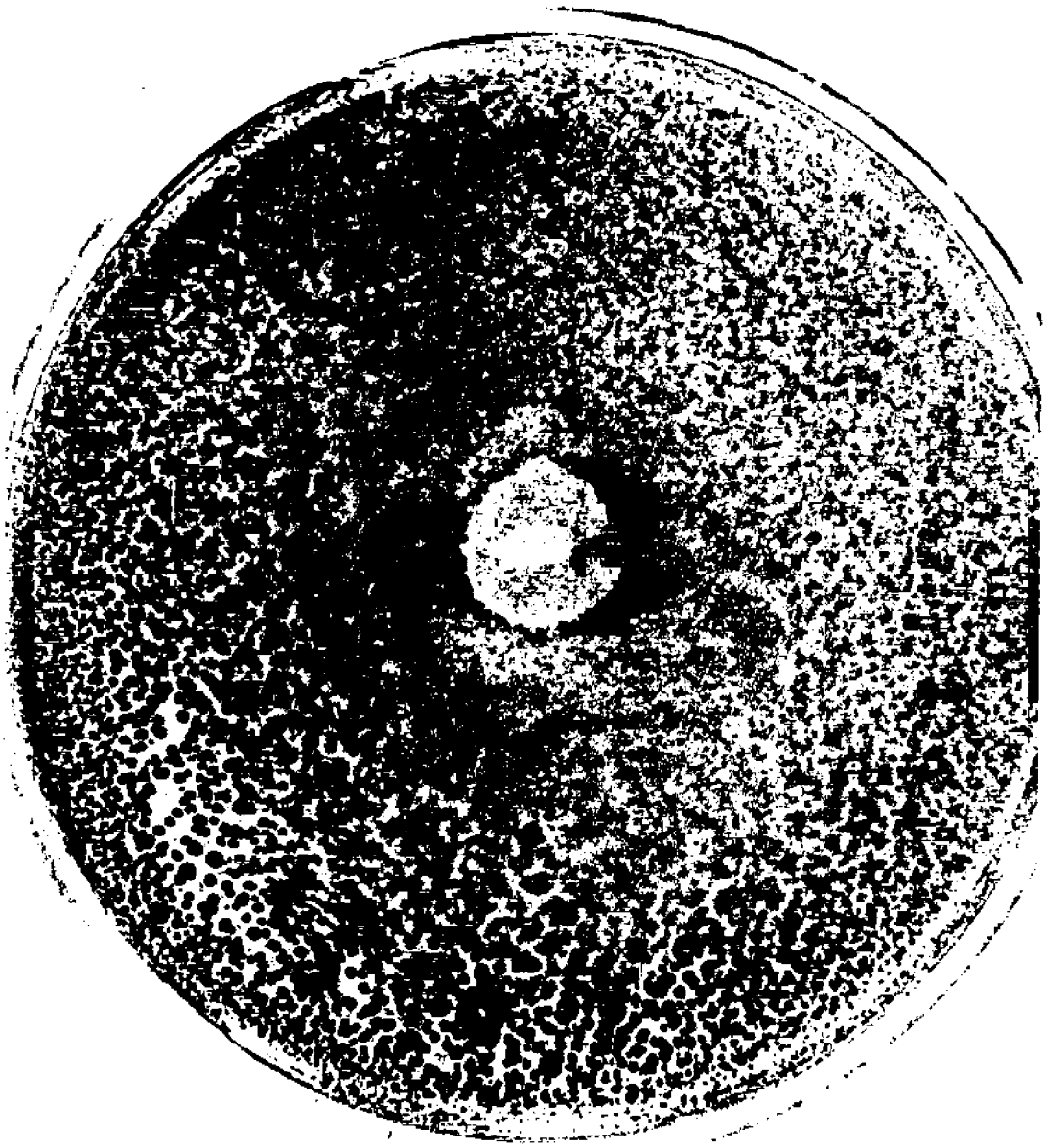

FIGURE 1. Host susceptibility testing using the spot test method. The clearing in the center of the petri dish is a result of lysis of the V. parahaemolyticus host lawn by Bdellovibrio strain OC1.

TABLE 4. Quantification of marine bdellovibrios from surface ocean water samples at Ocean City, Maryland during 1976-1978.

Month	Temperature[a] (°C)	Salinity (ppt)[b]	pfu Plate 1	pfu Plate 2	Mean
1976					
June	17.2	32.6	24	36	30
October	20.5	32.1	188	252	220
November	10.6	31.0	6	ND[d]	6
December	8.8	31.9	1	3	2
1977					
January	1.1	30.3	0	0	0
February	0	32.1	0	ND	0
April	11.6	31.0	0	0	0
May	18.8	31.6	20	25	23
August	20.0	32.5	170	60	115
September	21.6	32.1	18	30	24
October	18.3	32.5	2	6	4
December	7	30.3	123	ND	123
1978					
February	2	-	0	2	1
March	5	-	0	0	0
May	-[c]	-	12	18	15
September	22	-	0	4	2

[a] Mean temperature for each month from National Ocean Survey, Rockville, Maryland

[b] Mean salinity for each month from National Ocean Survey, Rockville, Maryland

[c] Datum not obtained.

[d] Not done.

TABLE 5. Quantification of marine bdellovibrios from surface water at Solomons Island and Annamessex River during 1977-78.

Solomon's Island				
Month	Temperature (°C)	Salinity (ppt)	pfu/10 ml Plate 1	Plate 2
November, 1977	16	9.3	148	245
January, 1978	1	8.8	0	0
May, 1978	15	7.5	16	14
September, 1978	26	13.1	1500	ND[a]

Annamessex River				
Month	Temperature (°C)	Salinity (ppt)	pfu/10 ml Plate 1	Plate 2
August, 1977	-[b]	18	15	ND
January, 1978	1	11	4	10
March, 1978	6	13	2	1
April, 1978	16	13	650	ND
July, 1978	24	14	520	524
September, 1978	23	14	3300	ND

[a] Not done

[b] Datum not collected

per 10 ml of water sample. Also shown are the temperatures and salinities of the water samples when they were recorded for samples at Ocean City; however, the salinity range in the ocean water remained relatively constant in comparison to the more varied salinities recorded at the Crisfield and the Solomons Island stations. The pH at all stations remained fairly constant between pH 7.5 to 7.9. Higher numbers of marine bdellovibrios were observed in the late summer and early fall months at all the stations. There were very few bdellovibrios observed in the winter months of December, January, February and March, with the exception of one sample taken at Ocean City in December following a storm. The highest counts recorded suggested a 1 to 2 log higher number of bdellovibrios at the Chesapeake Bay stations than in the ocean water. High counts of bdellovibrios were usually observed in the water samples when the highest water temperatures were recorded.

Since June, 1979, monthly samples of top and bottom water from the Patuxent River have been collected at three different locations off the pier of the Chesapeake Biological Laboratory at Solomons Island. The collected water samples were cultured for marine bdellovibrios, TVC and total count on TCBS agar. The data collected thus far (Table 6) confirms the seasonal distribution suggested in the earlier experiments (Tables 4 and 5). The numbers of marine bdellovibrios recovered from both top and bottom water samples were higher in the warmer months and decreased almost to zero in January. The TVC and the total counts on TCBS medium also showed a decrease in the number of bacteria during the colder months.

TABLE 6. Recovery of marine bdellovibrios from top and bottom water samples collected from the Patuxent River at three different locations off the pier of the Chesapeake Biological Laboratory.

Month	pfu [a]/10 ml of water sample	TVC/ml x 10^2	TCBS/ml x 10^1
June	19.3	-[b]	-
July	104.6	-	-
August	55.5	9.4	6.2
September	39.4	9.8	-
October	45.6	89.8	5
November	30.0	97.6	35.2
December	24.3	38.2	12.8
January	1.0	56.7	0.8

[a]= Average of triplicate plates of top and bottom water samples from three different locations on the pier.

[b]= No counts available.

Distribution of Marine Bdellovibrios in the Chesapeake Bay and James River. The results of quantifying the number of bdellovibrios in water samples taken along the Chesapeake Bay and from the James River in 1978 are shown in Tables 7 and 8 and Figure 2. The average number of colonies are shown for halophilic bdellovibrios, V. parahaemolyticus-like organisms (VPLO), Vibrio-like organisms (VLO), and for total aerobic heterotrophs. Other data collected and shown in Tables 6 and 7 include water temperature, salinity, dissolved oxygen, transparency and station depth.

The highest bdellovibrio counts were recovered from the mid-lower regions of the Chesapeake Bay with low counts recovered from both the northern and southern extreme ends of the Bay. Within the regions of the Bay where high counts were recovered, these counts were obtained from both top water samples and bottom water samples. The two highest bdellovibrio counts were collected at Smith Point and at the York River in top water samples. However, at the Rappahannock River, no pfu were recovered from the top water sample, but a count of 299/10 per ml of sample was obtained from bottom water samples. The results show no obvious correlation between pfu and bacterial counts on thiosulfate citrate bile salts (TCBS) agar and total counts of aerobic heterotrophs on sea water yeast extract (SWYE) agar. For example, the total counts on SWYE at the Newport News and Little Creek stations on the James River were similar in both top and bottom water samples. The water temperatures at Smith Point and York River stations were similar and the salinities were fairly close. However, a comparison of the

TABLE 7. Recovery of marine bdellovibrios from various sites in the Chesapeake Bay.

Station	Station depth (meters)	Sample depth (meters)	Temperature (°C)	Salinity (ppt)	Dissolved oxygen (ppm)	Turbidity (feet)	Plaque-forming units/ 10 ml sample	VPLO & VLO[a] colony-forming units	Total count in colony forming units
Jones Falls	7.9	1	22.44	4.8	-[b]	1	0	9.0×10^3	3.5×10^4
		6.9	22.67	4.6	-		4	2.4×10^4	1.3×10^5
Colgate Creek	8.8	1	22.21	8.0	-	0.75	3	3.0×10^3	1.0×10^4
		7.8	22.12	8.1	-		1	6.9×10^4	1.0×10^5
Chester River	10.3	1	20.48	4.5	-	1	0	0	1.0×10^2
		9.2	17.93	10.6	-		10	6.2×10^1	1.0×10^3
Smith Point	21	1	25.43	10.4	9.5	1.6	1594	3.6×10^2	1.28×10^3
		19	23.27	17.2	2.6		47	9.0×10^1	7.0×10^2
Rappahannock	11.2	1	26.00	12.5	8.8	1.2	0	0	1.23×10^3
		10.0	23.42	20.3	2.9		299	1.35×10^3	1.3×10^3
York River	10.6	1	26.75	15.4	9.3	3.3	580	1.1×10^3	7.0×10^2
		9.7	22.91	23.3	4.4		53	0	0
Cape Henry[c]	13.7	1	24.01	23.6	8.2	1.9	2	1.9×10^2	5.1×10^2
		12.7	15.85	31.8	6.7		1	2.0×10^2	1.5×10^2

[a] *V. parahaemolyticus*-like organisms and *Vibrio*-like organisms.

[b] Datum not available.

[c] Only sediment sample collected was at this station. Bdellovibrio count from 5 ml of sediment supernatant was 205.

TABLE 8. Recovery of marine bdellovibrios from sites in the James River.

Station	Station depth (meters)	Sample depth (meters)	Temperature (°C)	Salinity (ppt)	Dissolved oxygen (ppm)	Turbidity (feet)	Plaque-forming units/ 10 ml sample	VPLO & VLO[a] colony-forming units	Total count in colony forming units
Little Creek	7.6	1	25.28	20.1	8.0	1.8	1[b]	1 x 10^1	2 x 10^3
		6.3	22.74	22.6	5.6		2	2 x 10^1	8.0 x 10^2
Newport News	11.3	1	26.28	15.8	8.4	1.0	11	4.7 x 10^2	1.25x 10^3
		10.3	25.20	19.4	7.1		20	1.2 x 10^1	5.5 x 10^2
Sloop Point	19.8	1	28.43	0.2	7.2	0.4	0	0	1.1 x 10^2
		-[c]	27.48	0.4	6.9		0	1 x 10^1	2.6 x 10^2

[a] *V. parahaemolyticus*-like organisms and *Vibrio*-like organisms.

[b] Mean pfu for 2 plates.

[c] Datum not obtained.

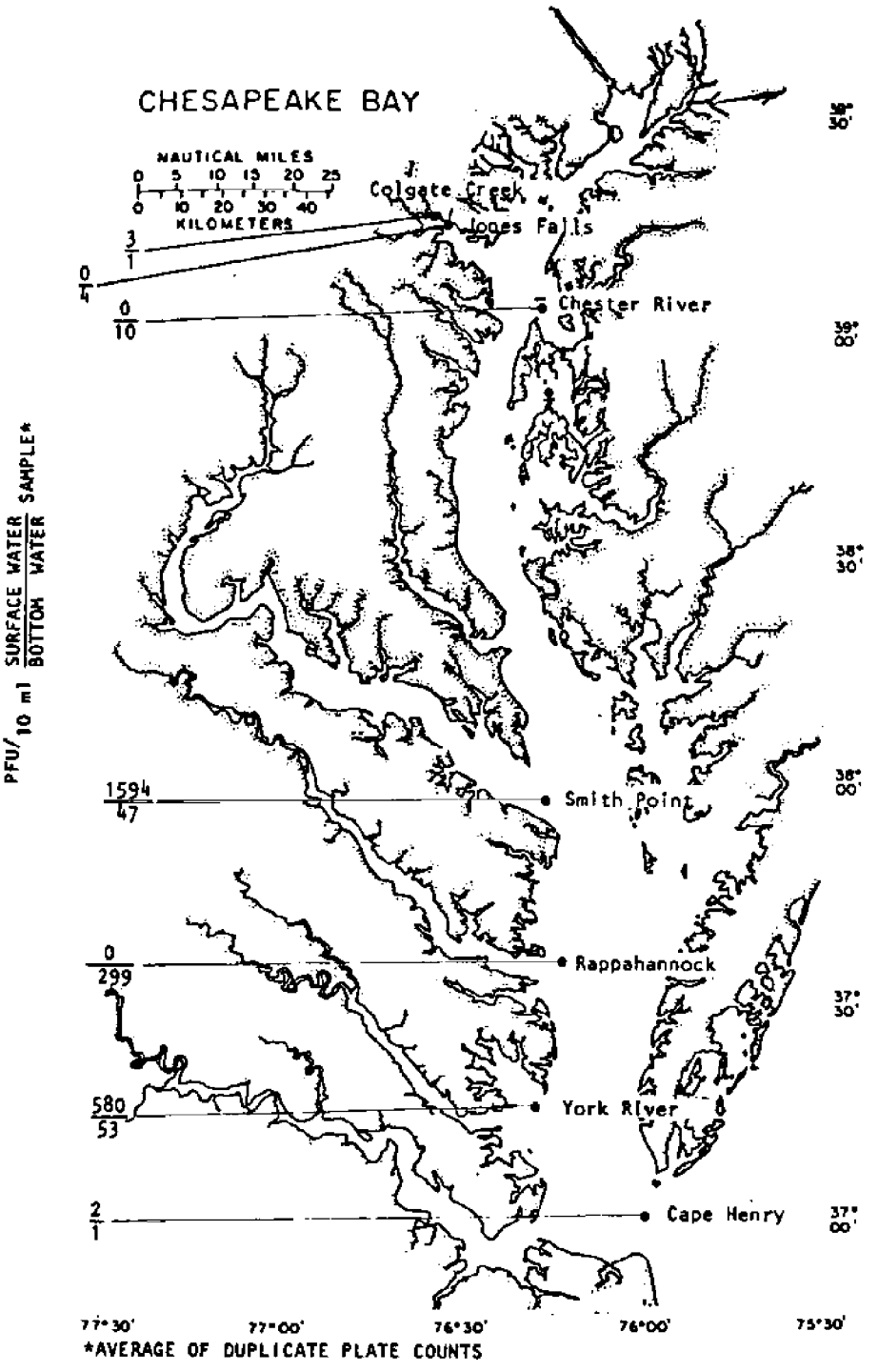

FIGURE 2. Map of the Chesapeake Bay showing sampling stations. Numbers on the left refer to numbers of bdellovibrios recovered from top and bottom water samples at various stations, as listed in Tables 7 and 8.

bdellovibrio counts at the two stations showed a marked difference of 2 logs in the number of organisms, the highest being at Smith Point.

Bdellovibrio counts were obtained over a wide range of salinities with the higher counts occurring between 10.4 parts per thousand (ppt) at Smith Point to 20.3 ppt at the Rappahannock River station. Few bdellovibrio pfu were recovered from stations where the salinity measured less than 20.0 ppt. Also, few bdellovibrio were recovered from the station having the highest salinity measurement, Cape Henry. Relatively high bdellovibrio counts occurred over a wide dissolved oxygen range as seen at the top sample at the York River (9.3 parts per million (ppm)) and the bottom sample at the Rappahannock (2.9 ppm).

The number of bdellovibrios recovered in the James River was lower than counts recovered from the Chesapeake Bay even when the physical and chemical factors measured were relatively similar. The temperatures at all stations sampled were within a close range of each other, with the exceptions of the bottom water temperatures at Cape Henry and at the Chester River. The highest temperatures were recorded at the James River stations. The James River stations presented a salinity gradient with decreasing salinity from the Little Creek Station to the Sloop Point Station.

In June 1979, a second study on the distribution of marine bdellovibrios in the Bay was conducted in a similar manner as the initial study discussed above. Again, the highest bdellovibrio counts were recovered from the

TABLE 9. Distribution of marine bdellovibrios in the Chesapeake Bay, June, 1979.

Station	Station depth (m)	Sample depth (m)	Temperature (c)	Salinity (ppt)	pH	Dissolved oxygen (ppm)	Tubidity (m)	PFU/[a] 10 ml sample	TCBS counts	Total count (SWYE)
Tolchester Beach	6.7	1.0	21.68	2.45	7.43	-[b]	1.0	0	1×10^1	1.1×10^3
		5.7	21.57	3.43	7.36			0	1.5×10^1	9.2×10^2
Chester River	11.3	1.0	20.95	5.39	7.40	6.4	1.2	0	-	6.0×10^2
		10.0	19.61	10.84	7.18			1	-	1.2×10^3
Cooks' Point (Choptank)	8.23m	1.0	21.57	8.34	7.22	8.0	2.0	34	-	1.6×10^3
		7.2	20.45	12.35	7.32			197	-	-
Patuxent River	12.8	1.0	22.10	9.24	8.34	8.9	1.7	2156	1.6×10^2	1.3×10^4
		11.8	21.42	10.36	7.76			2076	-	-
Smith Point	36.58	1.0	22.60	10.20	8.37	9.4	2.1	25	4.9×10^2	3.1×10^3
		30.0	22.04	17.40	7.65			335	5.0×10^1	3.8×10^2
Rappahannock River	11.58	1.0	22.46	12.43	8.3	8.2	2.2	210	4.6×10^2	1.8×10^3
		10.4	21.84	17.90	7.65			83	2.4×10^2	5.5×10^2
York River	10.67	1.0	21.69	16.04	8.06	7.9	2.5	2	1.3×10^2	4.7×10^3
		9.6	21.02	24.43	7.87			8	1.6×10^2	1.0×10^3
Cape Henry	16.76	1.0m	21.15	19.99	8.15	8.4	11.6	2	9×10^1	8×10^2
		15.76	19.80	27.57	7.98			6	1.5×10^2	3.8×10^2

[a] Plaque forming units - represents average count from two samples, each of which was plated in triplicate.

[b] Datum not available.

mid-region of the Bay extending from Cook's Point at the mouth of the Choptank River to the Rappahannock River (Table 9). The highest counts were recovered just upstream from Smith Point at the mouth of the Patuxent River. The measurements of the physical and chemical parameters of the water samples again showed no correlation with the number of marine bdellovibrios recovered, with the possible exception of salinity. The summary of the distribution of marine bdellovibrios in the Bay as observed in the two years in which the study was conducted is shown in Figure 3.

Recovery of marine bdellovibrios from selected habitats: oyster shell epifauna, marine rope and fish slime.

Oyster shell epifauna was collected from oysters contained in oyster baskets suspended from the pier at the Chesapeake Biological Laboratory at Solomons Island. An amount of epifauna sufficient to increase 20 ml of sea water to a volume of 25 ml was collected from two oysters. Material from the surface of marine rope submerged in the water was collected in the same manner. Within 30 min, the tubes containing the collected specimens were mixed by vortexing for 1 min and were then placed in a rack and left undisturbed for 1 h. The supernatant fluid was then aspirated with a pipette and cultured for marine bdellovibrios, total viable counts (TVC) and total counts on TCBS. The oyster shell epifauna continued to be cultured on a periodic basis as shown in Table 10. The number of bdellovibrios recovered ranged from 4.5×10^1 to 2.4×10^4 pfu/ml in samples collected

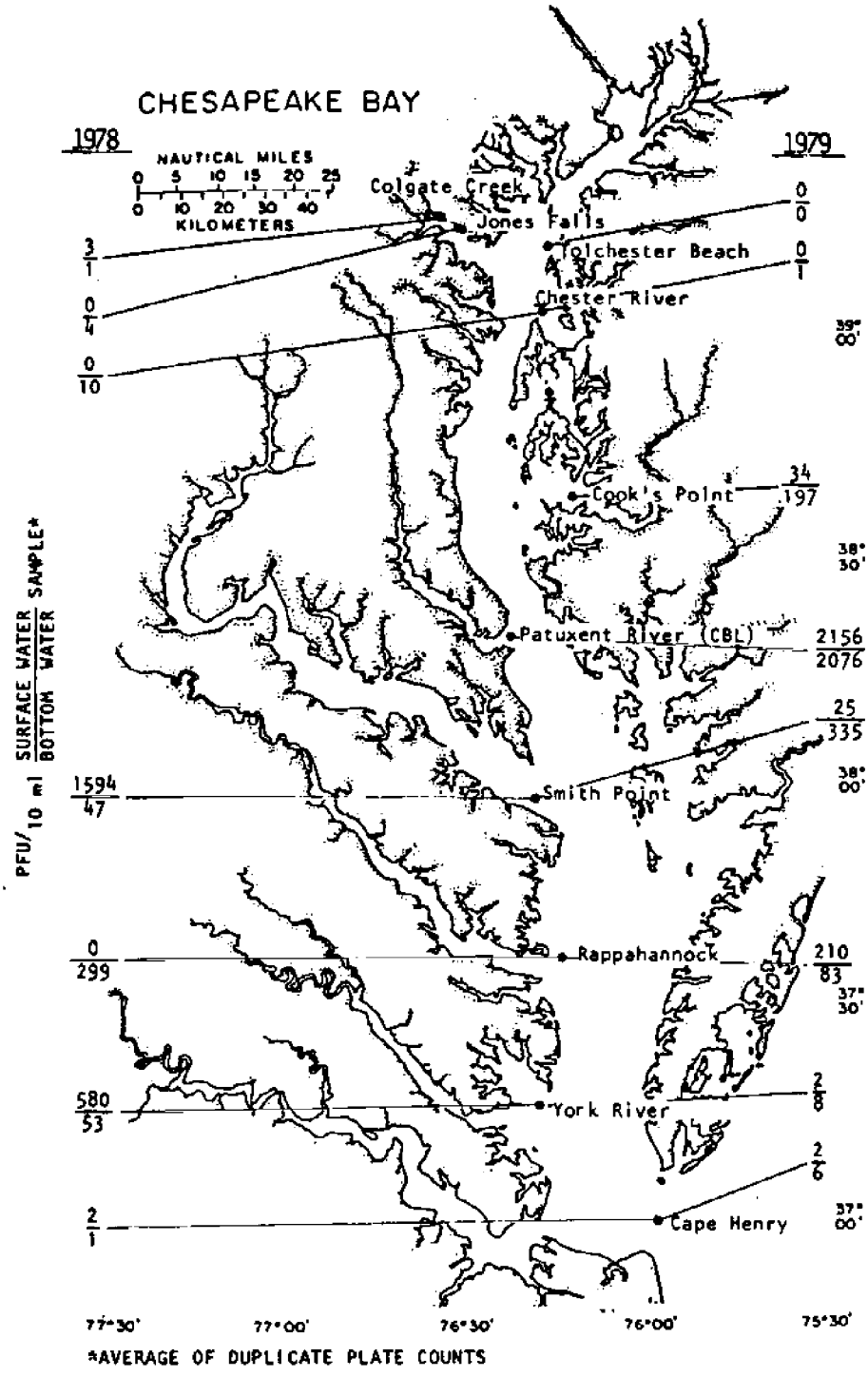

FIGURE 3. Map of the Chesapeake Bay showing sampling stations. Numbers refer to numbers of bdellovibrios recovered from top and bottom water samples at various stations listed in Table 9.

TABLE 10. Recovery of marine bdellovibrios from oyster shell epifauna.

Month		M. Bd. pfu/ml	TVC/ml	TCBS/ml
June		1.4×10^3	2.0×10^3	3.0×10^3
July		5.0×10^3	-	-
September	Spec. #1	6.0×10^3	5.9×10^5	0.1×10^4
	Spec. #2	2.4×10^4	6.5×10^5	3.1×10^5
January		2.57×10^2	4.12×10^7	8.50×10^6
February		4.5×10^1		

between June, 1979 and February, 1980. The TVC and the number of organisms recovered on the TCBS plates are also shown in Table 10. The number of bdellovibrios recovered appears to be higher in the warmer months than in the colder months, which is consistent with the counts observed from water samples in warm and cold months.

The surface of submerged marine rope was cultured at the same time as the oyster shell epifauna in June, 1979. The rope surface sample was observed to have bdellovibrio counts similar to those observed from the oyster shell epifauna. The bdellovibrio count from the supernatant fluid of the rope scrapings was 1.7×10^3 pfu/m. The TVC was 1.4×10^5 organisms/ml and the count on TCBS medium was 6×10^3 organisms/ml of supernatant fluid.

Small fish (Blemys) measuring 3-5 in. in length were often found in the oyster baskets. These were caught and placed in a container of 25 ml of sterile ocean water. The surface of the fish was scraped with a spatula and the scrapings were placed in 10 ml of sterile sea water. The suspension was vortexed and cultured for marine bdellovibrios, TVC and TCBS. Bdellovibrio counts from specimens taken one week apart were 3.0×10^2 pfu/ml of supernatant fluid (6/9/79) and 1.4×10^2 pfu/ml (6/15/79) respectively. The specimens collected on June 15 were also cultured for TVC and counts on TCBS. The TVC was 2.7×10^6 and the count on TCBS was 4.3×10^5.

Bdellovibrio antigen preparations. Bdellovibrio strains OC1, 110, and BM11 were grown in

HS broth on host cells of V. parahaemolyticus (P-5), Enterobacter cloacae (ATCC 15361) and Pseudomonas (LR-101), respectively. After clearing of the culture fluid, the bdellovibrios were separated from the host cells by centrifugation at 10,000 x g for 20 min. The bdellovibrio cells in the supernatant fluid were then concentrated by centrifugation at 18,000 x g for 30 min. The pelleted organisms from the high speed centrifugation were resuspended in sterile sea water (two ml) and centrifuged in a 5 to 25% ficoll (Sigma) gradient in sterile sea water. After centrifugation at 4,000 x g for 30 min, the bdellovibrio band was removed, resuspended in 2 ml sterile sea water, quantified for bdellovibrios by direct count and repeatedly frozen and thawed 12 times. The protein concentrations of the antigen preparations were determined by the method of Lowry (31) using bovine serum albumin (Calbiochem) as a standard.

Preparations of rabbit antisera to bdellovibrio strains OC1 and 110. At least two white male New Zealand rabbits (3 to 4 kg) were injected twice subcutaneously in the back (with a one-week interval between injections) with 1 mg of protein (0.5 ml) of the antigen preparations of each strain in 0.5 ml Freunds's complete adjuvant (Difco). These injections were followed by two intraperitoneal injections of 1 mg of protein in 1 ml of sterile saline of the antigen preparation at weekly intervals. After seven days, the rabbits were bled via the marginal ear vein and the prepared sera stored at -20° C until used. In addition, several rabbits were given booster injections of 1 mg of protein of the antigen preparations intraperitoneally. Serum prepared to strains OC1 and 110 were

absorbed with 40% suspensions (determined from packed volumes resulting from centrifugation at 600 x g for 20 min) of the host bacteria used for the growth of the antigen preparations.

Immunodiffusion (ID) and immunoelectrophoresis (IE). Double diffusion in two dimensions was performed on glass slides using 1% agarose (Calbiochem) in 0.05 M borate-buffered saline, pH 9.0. Glass slides overlaid with 3 ml of the 1% buffered agar gel were used for IE (37). The slides containing the antigen preparations were electrophoresed in Veronal Buffer, pH 8.6 (37). Antigen preparations were teted at concentrations of 1 mg protein per ml as well as two-fold serial dilutions of this concentration. Serum was tested undiluted or in serial two-fold dilutions.

Antigenic reactions of strains OC1, BM11 and 110 when tested by immunodiffusion (ID) and immunoelectrophoresis (IE) with rabbit anti-OC1 and anti-110 sera. The antigenic preparations (AP) of marine strains OC1, BM1, and terrestrial strain 110 were reacted by ID and IE with rabbit anti-OC1 and anti-110 host-absorbed sera. During ID experiments, at least two precipitin lines were observed upon reaction of anti-OC1 serum and strains OC1 and BM1 and by anti-110 serum and strain 110. One of these precipitin lines was a sharper line equidistant between the antigen and serum wells while the other precipitin line was concave toward and in close proximity to the antigen well. There were no reactions upon testing the anti-110 serum with strains OC1 and BM11 AP or with anti-OC1 serum and strain 110 AP when several dilutions of the

AP and serum were tested. Similarly, no reactions were observed with the antisera upon testing them with AP of the host bacteria used to grow the bdellovibrio strains. Lines of identity were observed with strain OC1 and the Israeli strain BM11 when several dilutions of the AP were used. IE with homologous systems resulted in the production of a sharp line which was concave towards the antigen well and which demonstrated no apparent charge migration. Again, no heterologous reactions were observed with IE testing.

DISCUSSION

Although marine bdellovibrios have been isolated from Maryland coastal and Chesapeake Bay waters, this is the first study of their occurrence and distribution in these waters. Quantifying recovery of marine bdellovibrios from water samples taken at various sites on the Bay showed that although bdellovibrios can be recovered from all parts of the Bay, they appear in greatest numbers in the mid and lower regions. In the northern parts of the Bay (Chester River, Colgate Creek and Jones Falls), where salinity is less than 1‰, bdellovibrios were recovered in numbers less than 1 pfu per ml. In the lower regions of the Bay (Smith Point, Annamessex River, York River, and Rappahannock River), where salinity is greater than 1‰, the highest numbers of bdellovibrios (10^2/ml) were recovered. However, at Cape Henry where salinity reached 31.8 ppt, the bdellovibrio count dropped, indicating a salinity range for these organisms.

Recovery of marine bdellovibrios from Chesapeake Bay and Atlantic Ocean waters is dependent too on the season that sampling is undertaken. In the warmer months the number of bdellovibrios recovered was 10^2 to 10^3 logs higher than the number recovered in the colder months. Very few bdellovibrios were observed from December through March, except in an ocean sample taken following a storm. Bay water contained higher numbers of bdellovibrios than ocean water, and highest counts were obtained at the highest water temperatures. This is the first report of a seasonal fluctuation in the

number of marine bdellovibrios recovered from marine waters.

Previous investigators have used methods which caused fewer numbers of bdellovibrios to be recovered. Using the direct culture technique and polypeptone 20 medium, bdellovibrios were recovered in higher numbers per volume of Chesapeake Bay water than in any previous reports for any water samples. The results suggest that direct culturing is the method of choice for quantifying bdellovibrios in water samples.

In tests of host susceptibility to bdellovibrios, cultural studies revealed that plaque sizes are larger with all hosts tested when natural sea water as opposed to artificial sea water is used in the preparation of media. Larger plaques formed with polypeptone 20 medium than with the five other media tested. The results suggest susceptibility-testing media should include: host sea water, polypeptone 20, and nutrient broth 20 media. Additionally, the type of media used affected the susceptibility of host bacteria to infection by marine bdellovibrios. Using a multi-media testing system demonstrated that the marine bdellovibrio isolates had a wide range of susceptible hosts.

In previous studies marine bdellovibrios have been isolated and quantified only from water samples. This is the first report of their isolation from the surfaces of marine animals and submerged nonvital material. Bdellovibrios appeared in higher numbers on oyster shell surfaces than in water samples near the

oysters, which suggests their activity is an ecological factor in marine life.

Studies of the antigenic properties of marine bdellovibrios, never attempted before, showed that the marine strains did not share antigenic determinants with the terrestrial strain tested. But a marine strain from Maryland coastal waters did share antigens with a marine strain isolated from the Mediterranean coast of Israel.

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