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Effects of Temperature, Salinity, and Substrate on the Colonization of Surfaces In Situ by Aquatic Bdellovibrios

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Recent studies suggest that surfaces are a more conducive habitat than the water column for the proliferation of bdellovibrios in the aquatic environment. The effect of temperature and salinity on the colonization of bdellovibrios on oyster shell, glass, and polystyrene surfaces in situ was investigated over an annual cycle. Sterile surfaces were suspended in various bodies of water for intervals ranging from 24 to 120 h. The results revealed that bdellovibrios associated with different types of surfaces over a broad temperature and salinity range. After 24 h of submersion in waters with temperatures from 9.0 to 26.7°C, the ranges in $\log_{10}$ values per square centimeter for the three surfaces were as follows: oyster shell, 2.2 to 2.5; glass, 0.3 to 2.2; and polystyrene, 0.7 to 1.6. Bdellovibrios were not recovered from surfaces submerged in water at temperatures below 8°C during the 120-h experimental cycle. The number of bdellovibrios and culturable bacteria on oyster shells was significantly higher than the numbers on glass and polystyrene at all time intervals. The number of bdellovibrios was positively correlated with temperature and salinity on all surfaces. A positive correlation between the number of recoverable bacteria and temperature was observed, but the results with respect to salinity were diverse. The numbers of bdellovibrios recovered from oyster shells (up to 48 h) and water samples were significantly increased at salinities greater than 11‰ compared to those in lower-salinity environments. The results of this study reveal that like many other bacteria in the aquatic environment, bdellovibrios prefer to associate with surfaces. This association provides the predators a rich source of prey bacteria in surface biofilms and perhaps protection in the gel-like matrix of the biofilm.

Bdellovibrio bacteriovorus is a small (0.2-μm), comma-shaped, gram-negative, predatory procaryote which obligately preys on many gram-negative bacteria (6, 16, 17, 21). The characteristic lethal attack by bdellovibrios on prey bacteria has generated interest in its impact on bacterial mortality in nature and its potential as a biological control agent. However, the low numbers of bdellovibrios typically recovered from the water column and sediment (25, 27), the sites most often studied, have established some doubt about the influence of the predators on populations of bacteria in the aquatic environment. In response, it has been suggested that both the number and activity of the bdellovibrios may be higher in habitats other than bulk water and sediments (13). Recent studies have revealed that greater numbers of the predators occur at the air-water and solid-water interfaces (24, 29).

Surface colonization may be an important strategy in the ecology (28) of the bdellovibrios and an important mechanism of their survival in the aquatic environment, especially oligotrophic waters. The surface biofilm matrix offers these predators large numbers of potential prey bacteria and perhaps some protection from environmental forces, thus making the colonization of surfaces beneficial for the bdellovibrios. To maximize this benefit, the predators would need the capability to colonize many types of surfaces under different environmental conditions. The data from previous studies in this laboratory have revealed that water temperature has a direct influence on the number of bdellovibrios recovered from the water column and sediment (25, 26). A preliminary investigation has revealed that temperature may affect the association of the predators with some surfaces (28, 31). The study of the physical, chemical, and biological variables of bdellovibrio colonization of surfaces is essential to broadening our understanding of the ecology of these predatory bacteria. The influence of a wide range of temperature and salinity conditions on the colonization by bdellovibrios of different types of surfaces in situ was investigated. In the context of this study, association with surfaces refers to the presence of bdellovibrios on surfaces, with no distinction whether the predators are attached directly to the substrate surfaces or to surface-associated bacterial cells or embedded in biofilms. Also, it is recognized that in some cases the presence of bdellovibrios and other species on surfaces is the result of a new association or multiplication of those organisms previously associated with the surfaces.

MATERIALS AND METHODS

The major part of this study was conducted in the Patuxent River, a tributary of the Chesapeake Bay (30°19′N, 76°27′W), from April 1992 through April 1993. A second study site was on the Gunpowder River (39°22′N, 76°18′W) in September and October 1992, June 1993, and October 1993. A third site was the coastal water (Caribbean Sea) off La Parguera at the southwestern corner of Puerto Rico (17°59′N, 67°03′W) in May 1994.

The surfaces selected for this study were oyster shells, glass, and polystyrene. Oyster shells were selected because oysters (*Crassostrea virginica*) are indigenous to much of the Chesapeake Bay area and were the surfaces initially observed to harbor large numbers of bdellovibrios (29). Glass contains silica, a natural component of sediments and shore sands. Polystyrene, as well as glass, may be useful in future studies for observing attached bacteria by light microscopy if it is found to be an adequate surface for colonization.

Preparation of test surfaces. The oyster shells used in this study were selected based on similarity in size and topography of the surface. Each shell was numbered for identification purposes, measured by the foil overlay method (22) to approximate the outer surface area, scrubbed to remove extraneous material, and drilled to make a 2-mm hole. Each set of duplicate shells was secured at 10-cm intervals to 4 m of twine threaded through the drilled holes.

Glass slides were buffed with coarse sandpaper and secured in a glass slide rack with rubber bands, and the rack was tied to twine. The oyster shells and the slide rack with slides were placed in individual paper wrappers and autoclaved for 15 min.

The polystyrene surface used in this study consisted of sterile disposable culture dishes (100 by 15 mm) (4). To secure the dishes for submersion in the

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river, a 1/4-in. loop-top zinc screw was inserted into the top and bottom pieces, respectively. The same line to which the oyster shells were attached was threaded through the loop-top screw so as to position one of the halves 10 cm above the shells prior to deployment.

Experimental protocol. From the research pier at the Chesapeake Biological Laboratory, Solomons, Md., each of the test surfaces, in multiples of eight, was suspended in the water column approximately 30 to 50 cm above the floor of the Patuxent River over a 120-h period. The depth at this site typically ranged between 2.0 and 2.5 m. Test surfaces in duplicate were retrieved at intervals of 24, 48, 72, and 120 h. At the lowest temperatures, two additional oyster shells were suspended and remained in place for 14 days. This was done because preliminary data at lower temperatures indicated a decreased rate of colonization (28, 31). The same oyster shells were used in each experiment, and the identical shells were always retrieved at the same time (24 to 120 h) interval. Upon retrieval, each test surface was drained for 30 s, placed in a covered sterile petri dish to prevent drying, and transported to the laboratory. Simultaneously with the retrieval of surface specimens, a 50-ml sample from the water column was collected in a sterile container 3 cm (24) below the air-water surface directly above the location of the submerged surfaces. The elapsed time from collection to processing in the laboratory was 20 to 30 min. The samples were moistened with 5 ml of sterile artificial seawater (Instant Ocean; 26 g per liter of distilled water [pH 8.0]) upon arrival in the laboratory.

At the Chesapeake Biological Laboratory site, temperature and salinity measurements were obtained at the times when the surfaces were deployed in the water column and when they were retrieved; the measurements were made with a standard mercury thermometer and a hydrometer at a depth of 0.5 m. Measurements at the Gunpowder River site were taken with a model RS5-3 salinometer (Beckman Instruments, Inc.). Measurements at the Puerto Rico site were taken daily by staff personnel for the National Oceanographic and Aeronautical Administration; the instrumentation was unknown.

In the laboratory, each surface was brushed (8) or scraped (3), with intermittent rinsing with a total volume of 15 ml of artificial seawater. The resulting 15-ml suspension was pipetted into a centrifuge tube, mechanically agitated for 30 s with a Vortex-Genie mixer (setting 5) to deflocculate clumps (15), and centrifuged ($1{,}286 \times g$) for 3 min to sediment large particles, leaving bacteria in the supernatant fluid (3). Typically, 0.1 to 5 ml of supernatant fluid (undiluted or 10-fold dilutions) was plated (2) in duplicate onto polypeptone medium (26) for bdellovibrio PFU determination by the double-agar overlay method (18, 26). The prey used was *Vibrio parahaemolyticus* P-5 (12). Also, 10-fold serial dilutions of the supernatant fluid were spread-plated in duplicate onto estuarine agar (23) for bacterial CFU determination. Samples from the water column were cultured in the same manner as described above for the biofilm supernatant fluid. All plates were incubated at 25°C. After an initial incubation period of 5 days the polypeptone medium plates were examined daily over a 10-day period for plaque development. Plaques typical of those produced by bdellovibrios, clear with sharp boundaries and observed to increase in size (6, 24), were counted. The estuarine agar plates were examined daily for 7 days. Total plaque and colony counts were updated accordingly.

To measure the effects of temperature over an annual cycle, the experimental protocol was repeated at the Patuxent River site to coincide, to the greatest extent possible, with water temperatures of 0, 5, 10, 15, 20, 25, and 30°C. This approximates the typical yearly water temperature range of the Patuxent River at this location and was thought to provide sufficient increments to analyze the effects of temperature on bdellovibrio cultivation.

To observe the effects of high and low salinity on the association of bdellovibrios with surfaces, studies were conducted, respectively, from the shores of Magueves Island, La Parguera, Puerto Rico, and the Gunpowder River, a tributary of the upper Chesapeake Bay. At the Puerto Rico site, the sampling protocol of 24 to 120 h was followed, with an additional sample taken at 168 h. Only oyster shell surfaces and the water column were sampled. Deployment of the shell surfaces consisted of deploying a set of samplers on sequential days starting with the 168-h shells over a 7-day period and retrieving and harvesting all of the samplers on the same day immediately preceding the investigator's return to Baltimore. Plating of all samples was as described above, except that there was a 48-h delay due to the prey bacteria not being transported to the site because of restrictions on importing known pathogens into Puerto Rico. Because of the limited number of samples at this site and the 48-h delay in plating, the data were not included in the statistical analysis. In most experiments at the Gunpowder River, only the oyster shell surfaces and the water column were sampled. In one experiment, a set of shells was deployed for up to 168 h. In the two experiments where the water salinity was very low, the biofilm suspensions and water column samples were plated to recover nonhalotolerant (terrestrial or freshwater) bdellovibrios as well as the halophilic predators. For recovery of the nonhalotolerant bdellovibrios, plating was done on dilute nutrient broth with agar (14, 20) with *Escherichia coli* ML-35 as the prey organism. For recovery of the nonhalotolerant heterotrophic bacteria, plating was done on a low-salt medium, tryptic soy agar (Difco).

Statistical analysis. The statistical analyses were performed with the personal computer version of the Statistical Analysis System (SAS-PC 6.04) (11). First, all collected data were tested for normality. The data were subsequently subjected to logarithmic transformation to achieve a normal distribution, which also reduces the variation due to large standard deviations. All statistical analyses were

TABLE 1. Mean numbers of PFU and CFU recovered from various surfaces and water following submersion times up to 120 h[a]

Time (h)	No. of organisms (mean ± SD) recovered from:											
	Oyster shell			Glass			Polystyrene			Water		
	N[b]	PFU[c]	CFU[d]	N	PFU[c]	CFU[d]	N	PFU[c]	CFU[d]	N	PFU[e]	CFU[f]
24	15	8.8 ± 11.0	$1.1 \times 10^5 \pm 7 \times 10^4$	11	2.2 ± 4.5	$1.7 \times 10^3 \pm 3 \times 10^3$	10	0.9 ± 1.2	$5.4 \times 10^3 \pm 1 \times 10^4$	14	$3.0 \times 10^1 \pm 7 \times 10^1$	$7.8 \times 10^4 \pm 8 \times 10^4$
48	15	$2.8 \times 10^2 \pm 8 \times 10^2$	$1.1 \times 10^5 \pm 2 \times 10^5$	11	4.5 ± 5.8	$5.8 \times 10^3 \pm 7 \times 10^3$	9	3.8 ± 7.1	$4.4 \times 10^3 \pm 6 \times 10^3$	14	$2.6 \times 10^1 \pm 4 \times 10^1$	$7.6 \times 10^4 \pm 8 \times 10^4$
72	14	$4.4 \times 10^2 \pm 8 \times 10^2$	$1.1 \times 10^5 \pm 1 \times 10^5$	11	9.2 ± 20.7	$9.1 \times 10^3 \pm 1 \times 10^4$	9	4.2 ± 6.3	$7.1 \times 10^3 \pm 9 \times 10^3$	11	$2.7 \times 10^1 \pm 5 \times 10^1$	$5.0 \times 10^4 \pm 5 \times 10^4$
120	14	$9.4 \times 10^2 \pm 2 \times 10^3$	$1.4 \times 10^5 \pm 2 \times 10^5$	11	17.2 ± 24.3	$1.8 \times 10^4 \pm 2 \times 10^4$	9	15.6 ± 36.5	$1.0 \times 10^4 \pm 2 \times 10^4$	14	$2.8 \times 10^1 \pm 5 \times 10^1$	$7.3 \times 10^4 \pm 8 \times 10^4$

[a] The mean temperature was 14.6 ± 6.3°C (SD), with a range of 4 to 26.7°C ($N = 60$); the mean salinity was 10.4 ± 5.3‰ (SD), with a range of 0 to 16.5‰ ($N = 60$). Puerto Rico salinity data (35‰) were not included.
[b] Sample size.
[c] Bdellovibrio PFU per square centimeter on the test surface.
[d] Heterotrophic bacterium CFU per square centimeter on the test surface.
[e] Bdellovibrio PFU per milliliter.
[f] Heterotrophic bacterium CFU per milliliter.

based on the logarithmic transformed data set (5, 9) except for the analysis of the ratio of PFU per CFU.

To compare differences in the number of PFU or CFU recovered between oyster shell, glass, and polystyrene surfaces, the one-way analysis of variance (ANOVA), both unadjusted and adjusted for covariances (salinity and temperature), was used. The adjustments were made by using multiple-regression models. Furthermore, to detect the differences of PFU or CFU among three surfaces across all time intervals, repeated-measures ANOVA was used. The Bonferrioni *t*-test or Tukey's studentized range test was used for multiple comparison. The ratio of bdellovibrio PFU to bacterial CFU was calculated at the various time intervals for each surface, and a nonparametric test, the Kruskal-Wallis test, was used to estimate the difference between the surfaces. The Pearson product-moment coefficient (Pearson *r*) was implemented to determine the correlation between the numbers of PFU or CFU from each surface and the temperature and salinity. To reveal the impact of salinity on the number of PFU (or CFU) recovered from either oyster shells or water samples, the Student *t* test was performed (9).

RESULTS

The mean numbers of PFU and CFU recovered at 24, 48, 72, and 120 h from the surfaces of oyster shell, glass, and polystyrene submerged in the Patuxent River and the water column samples are shown in Table 1. The number of predators on all surfaces increased with time of submersion at all temperatures. In contrast, the number of predators in samples from the water column did not increase during the 120-h test period. The rate of bdellovibrio colonization on the different surfaces varied substantially. The association with the oyster shell surfaces (per square centimeter) increased greater than 30-fold from 24 to 48 h, whereas on glass surfaces the number doubled and on polystyrene surfaces the increase was approximately 4-fold. Following the 48-h interval, the colonization rate of the shell surfaces was reduced greatly, whereas on glass it remained nearly constant, doubling again from 48 to 72 h. Afterward, the rate decreased for both the oyster shell and glass surfaces, only doubling over the next 48-h interval between 72 and 120 h. The numbers associated with polystyrene changed little between 48 and 72 h but on average doubled daily between 72 and 120 h. The colonization of other bacteria as observed by increases in CFU per square centimeter on oyster shells proceeded rapidly between the time of submersion and 24 h. Following 24 h, however, the numbers remained relatively constant and the net rate of further colonization was essentially negligible and much lower than was observed with the bdellovibrios. The number of CFU recovered from glass surfaces increased over threefold between 24 and 48 h. The rate of accumulation then progressively decreased at the 72- and 120-h sampling intervals. The rate of increase on polystyrene was even lower.

Typically, the numbers of bdellovibrios recovered from all surfaces increased with temperature (warmer months versus colder months), reaching a maximum at 25.9°C (mean temperature over the 5-day experiment) (Fig. 1). As the temperature decreased to 7.5°C between August and December, a gradual decline in the number of bdellovibrios can be seen. At 4.5°C, the number of the predators was below the level of detection after the standard 120-h protocol (Fig. 1). However, following a more prolonged submersion (14 and 28 days) at comparable temperatures, bdellovibrios were recovered (6.4 and 29.0 PFU per cm^2, respectively). In the final sample of the study, which was collected during the spring warming when the temperature averaged 13°C, the numbers of bdellovibrios recovered from all surfaces increased substantially over that observed at temperatures less than 10°C (Fig. 1).

Since the temperature and salinity varied over a broad range (Table 1), adjustments of these two covariances in the statistical analyses were made. Tables 2 and 3 represent the unadjusted and adjusted one-way ANOVA results. However, when holding the covariances constant, the results of ANOVA re-

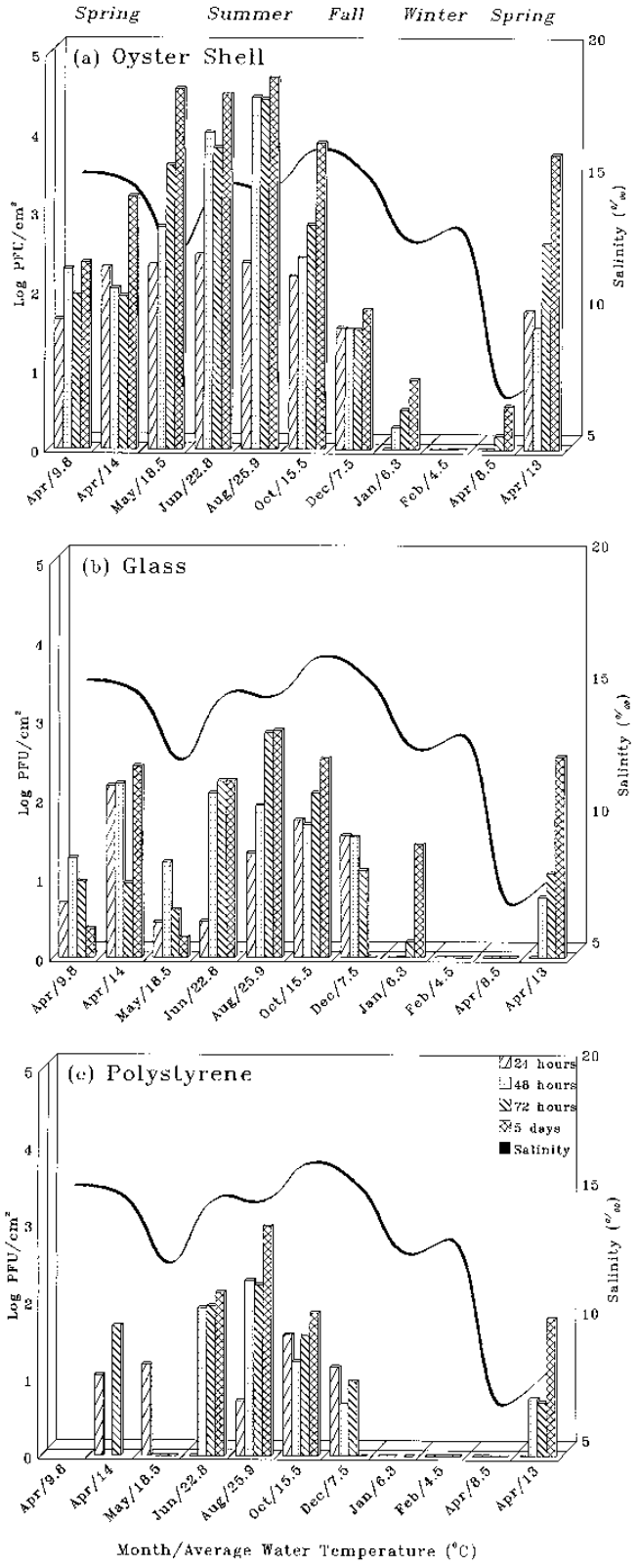

FIG. 1. Seasonal fluctuation of bdellovibrio PFU over an annual cycle in the Patuxent River with observed temperature and salinity as the variables for three surfaces: oyster shell (a), glass (b), and polystyrene (c).

TABLE 2. Results of one-way ANOVA of PFU among three surfaces[a]

Time (h)	Unadjusted		Salinity adjusted		Salinity and temp adjusted	
	F ratio	*P*	*F* ratio	*P*	*F* ratio	*P*
24	6.6	0.0044[b]	7.4	0.0026[b]	9.7	0.0007[b]
48	3.5	0.0433[b]	3.7	0.0375[b]	10.5	0.0004[b]
72	5.6	0.0091[b]	5.7	0.0084[b]	12.3	0.0002[b]
120	5.2	0.0119[b]	5.3	0.0114[b]	12.9	0.0001[b]

[a] For each measurement, two groups of surfaces were found, group A (glass and polystyrene) and group B (oyster shell). The different groups are significantly different at the 5% level, using the Bonferrioni *t*-test or Tukey's studentized range test.
[b] Significant at the 5% level.

vealed higher *F* ratios and lower probabilities among the surfaces (Tables 2 and 3). Coincidently, the repeated-measures ANOVA showed the same result when time was taken into consideration (PFU: $F = 5.78, P = 0.008$; CFU: $F = 12.92, P < 0.001$).

The correlation coefficients between measures of temperature or salinity and numbers of PFU or CFU are listed in Table 4. The numbers of both PFU and CFU on all surfaces were highly correlated with temperature. The correlation results between PFU and CFU and salinity varied according to the type of surface. A correlation was found between the numbers of bdellovibrios and salinities on glass and polystyrene but not on oyster shells. However, when salinity was ranked into two groups, above and below 11‰, respectively, and analyzed (Student's *t* test), the results revealed that the numbers of PFU from the oyster shell surfaces were significantly higher at the higher salinity at 24 and 48 h (respectively, $t = 2.93$ and 2.71, $P = 0.01$ and 0.023). In contrast, the numbers after 48 h were not significantly different. The CFU counts were not significant at any period. By using the same test, the numbers of PFU from water samples were significantly elevated at high salinity ($t = 4.69, P < 0.001$). The analysis of CFU in water column samples did not yield definitive information. The PFU/CFU ratios of three surfaces were not statistically different at any of the sampling intervals ($\chi^2 < 2.3, P > 0.3$) as determined by the Kruskal-Wallis test.

Bdellovibrios were observed to colonize surfaces submerged in bodies of water with extreme low and high water salinity measurements (Table 5). At the Gunpowder River site, with a salinity range of 3.4 to 3.8‰ in September 1992, halophilic bdellovibrios were recovered after 72 h from the submerged oyster shells but not from the glass surfaces. The initial rate of colonization was several log units lower compared to that of surfaces suspended in the higher-salinity (10 to 15‰) waters of the Patuxent River at a comparable temperature (see the data in Fig. 1a for the October sample). When the experiment was repeated in October with the salinity remaining in the same range, similarly low numbers of bdellovibrios were recovered (Table 5). In this experiment, the samples were also cultured for terrestrial bdellovibrios and none were detected. In June 1993, when the salinity measured zero, no halophilic bdellovibrios were recovered from the water column or the shell surfaces. However, cultures for the nonhalotolerant predators (which were done for only the 168-h sample) yielded high numbers of these organisms (Table 5). In October 1993, the salinity was at 4‰ and the numbers of halophilic bdellovibrios recovered from the oyster shell surface were higher than any of the numbers from the previous experiments at the Gunpowder River site and were approximately the same as those observed at the Patuxent River site at the same temperature, 15°C. No terrestrial bdellovibrios were recovered. When the PFU counts from shells submerged in the low-salinity waters of the Gunpowder River were included in the statistical analysis, the numbers of bdellovibrios recovered were significantly correlated with temperature and salinity (respectively, $r = 0.5, P < 0.001$; $r = 0.44, P < 0.001$).

At the Puerto Rico site, where the temperature (29°C) and salinity (35‰) are more stable, no predators were recovered from the water column (Table 5). However, bdellovibrios were recovered from the shell surface.

TABLE 3. Results of one-way ANOVA of CFU among three surfaces[a]

Time (h)	Unadjusted		Salinity adjusted		Salinity and temp adjusted	
	F ratio	*P*	*F* ratio	*P*	*F* ratio	*P*
24	8.4	0.0013[b]	8.4	0.0014[b]	16.1	0.0001[b]
48	7.3	0.0028[b]	7.5	0.0026[b]	14.9	0.0001[b]
72	9.2	0.0008[b]	9.0	0.001[b]	13.6	0.0001[b]
120	14.4	0.0001[b]	14.2	0.0001[b]	19.1	0.0001[b]

[a] See Table 2, footnote *a*.
[b] Significant at the 5% level.

DISCUSSION

In brackish and marine waters, halophilic bdellovibrios were observed to colonize different surfaces over a temperature range from 4 to 29°C and a salinity range of 3.4 to 35‰. The attraction of bdellovibrios to surfaces is shown by the fact that even when the predators were not detected in the water column (at the lower- and higher-salinity measurements), they were typically recovered from surfaces within a 24-h period and thereafter increased in number dramatically. The rate of association (presumed to include both attachment and multiplication) was observed to vary depending upon both temperature and salinity, being greater when these two parameters were high and reduced when they were low. Maximum association was observed at and above 18°C, minimal association was observed at or below 14°C, and no detectable colonization occurred below 5°C during the 120-h test period in which the study protocol was designed. However, after 14 days of submersion, bdellovibrios had colonized surfaces even at the lowest temperature (4°C). This suggests that the predators are capable of some activity at low temperatures. The numbers of bdellovibrios recovered from the surfaces were observed to increase as much as 100-fold over the 120-h period. An increase was not typically observed in the water column, revealing that the net increase in numbers was specific to the surface environment and was not a general phenomenon. Even at a temperature range between 5.5 and 7°C, increased numbers were observed on the oyster shell surface at 72 and 120 h.

The increase in the numbers of bdellovibrios at the surfaces was apparently due to both continued association and multiplication. Observations in our laboratory (unpublished data) reveal that increases in the numbers of predators occurred even after the surface had been removed from natural river water and relocated to sterilized river water that was free of predators or prey bacteria. Under these conditions, increases in bdellovibrio counts were most probably due to the organisms multiplying. This suggests that multiplication may account for most of the amplification observed in the natural environment.

Not unexpectedly, salinity appeared to exert some effect on

TABLE 4. Correlations between temperature and salinity measurements and numbers of PFU and CFU from three surfaces

Variable	Oyster Shell				Glass				Polystyrene			
	PFU		CFU		PFU		CFU		PFU		CFU	
	r[a]	P	r	P	r	P	r	P	r	P	r	P
Temp	0.81	0.0001[b]	0.66	0.0001[b]	0.61	0.0001[b]	0.46	0.0019[b]	0.66	0.0001[b]	0.56	0.0003[b]
Salinity	0.26	0.0861	0.09	0.5449	0.32	0.0367[b]	−0.06	0.7150	0.39	0.0181[b]	0.43	0.0074[b]

[a] Pearson *r*.
[b] Significant at the 5% level.

the colonization of the bdellovibrios to the shell surface. We were surprised, however, at the recovery of the halophilic predators from surfaces submerged in waters with a salinity measurement below 5‰. Previous reports (6, 19) indicated that bdellovibrios did not form plaques at salinities below 5.9‰. Colonization of the shell correlated with salinity. Also, the numbers of halophilic bdellovibrios recovered from surfaces in waters of 11‰ salinity or greater were statistically higher than the numbers from lower-salinity waters. When a salinity of zero was recorded at the Gunpowder River site, no detectable colonization of the surfaces by halophilic bdellovibrios occurred. However, the absence of salt was conducive for the surface colonization of the nonhalotolerant predators. This reveals that the attraction to surfaces is not limited to the halophilic predators.

Although bdellovibrios were recovered from all surfaces, the colonization of the oyster shells was the most dramatic. The greater number of CFU recovered from oyster shells undoubtedly was a major factor in the large numbers of predators recovered. The large number of potential prey organisms on the surface may influence the numbers of bdellovibrios in at least two ways. First, the large number of prey species increased the attraction of bdellovibrios to the surface such that more of the predators were drawn to the oyster surfaces than to the glass or plastic surfaces. Second, the larger population of potential prey probably contributed to a greater multiplication rate for the bdellovibrios. During the coldest months, the number of CFU on the various surfaces was observed to decrease by 0.5 to 1 log unit below that during the warmest months. Whether this contributed to the lower numbers of bdellovibrios recovered in the colder months is unclear, but we think that it is unlikely since we have observed declining numbers of

TABLE 5. Numbers of bdellovibrio PFU and heterotrophic bacterium CFU recovered from oyster shell and glass surfaces and water samples from the Gunpowder River and Puerto Rico study sites.

Sample trial[a]	Study interval	Oyster Shell		Glass		Water	
		PFU/cm^2	CFU/cm^2	PFU/cm^2	CFU/cm^2	PFU/ml	CFU/ml
GP-1	24 h	0.3	2.4×10^6	0.0		0.4	7.0×10^4
9/21–25/1992	48 h	4.0	4.5×10^5	0.0	1.2×10^4	1.4	4.7×10^4
16.2–21.0°C	72 h	1.9×10^2	1.1×10^6	0.0	1.3×10^4	0.7	3.0×10^4
3.4–3.8‰	5 days	—[b]	—	0.0	2.4×10^5	0.2	2.2×10^5
	5 days[c]			0.0	1.1×10^4	0.0	1.6×10^5
GP-2	24 h	0.1	9.8×10^3			0.0	1.2×10^5
10/6–10/1992	48 h	0.1	5.6×10^4			0.3	8.4×10^4
12.0–16.4°C	72 h	0.1	5.0×10^5			0.0	5.2×10^4
3.7–3.8‰	5 days	6.3	5.7×10^5			0.1	1.6×10^5
GP-3	24 h	0.0	3.5×10^5			0.0	1.1×10^4
6/10–21/1993	48 h	0.0	1.1×10^6			0.0	1.3×10^4
22.9–24.0°C	72 h	0.0	9.3×10^5			0.0	1.8×10^4
0.0‰	5 d	—	—			0.0	8.4×10^3
	7 d	0.0	4.8×10^5			0.0	1.0×10^5
	5 days[c]	—	—			2.2	8.4×10^2
	7 days[c]	3.6×10^3	7.4×10^4			5.2	1.6×10^3
GP-4	24 h	3.7	6.6×10^4			2.6	2.5×10^5
10/14–18/1993	48 h	9.1	2.0×10^5			2.1	2.0×10^5
15.0°C	72 h	—	—				
4.0‰	5 d	2.2×10^2	3.5×10^6			2.0	4.6×10^4
PR-1	24 h	0.6	1.1×10^4				
5/16–22/1994	48 h	2.0	1.1×10^6				
29.0°C	72 h	2.0×10^2	5.1×10^5				
35.0‰	5 d	4.9×10^1	3.8×10^4				
	7 d	6.1×10^2	1.3×10^6			0.0	5.7×10^2

[a] The submersion dates (mo/days/yr), temperature, and salinity are given for each sample trial.
[b] —, submerged surfaces were lost and were not available for culturing.
[c] Data in these rows are results of culturing for nonhalotolerant bdellovibrios on dilute nutrient broth with agar and heterotrophic bacteria recovered on tryptic soy agar.

predators when held at approximately 5°C even in the presence of excessive numbers of prey organisms (10^5/cm^2).

Another major factor in the large numbers of bdellovibrios on the shell was the rough topography of the shell surface, which may offer advantages for the initial association, persistence, and continued increase in the numbers of predators. The pitted surface with fissures may have provided some protection from the physical forces of the aquatic environment. We have observed in other studies that microorganisms tend to establish initial colonies in the ridges of surfaces before expanding to other areas (30). Other factors, including growth-promoting substances such as Ca^{2+} and Mg^{2+}, which are required by bdellovibrios (1), may be specifically adsorbed to the shell from the water. The components of the shell as it degrades in seawater may also be important. For example, chitin, which is found throughout the aquatic environment, has been reported to promote the growth of some *Vibrio* species (7). The fact that bdellovibrios can also colonize smooth surfaces is evidence of the diverse capabilities of these organisms and greatly expands their habitat range.

Although the numbers of bdellovibrios have been reported to fluctuate seasonally within the water column to below detectable levels during the colder months (25, 26), we have observed that the numbers within an established surface biofilm are relatively stable (29). This observation demonstrates the importance of surfaces and surface biofilms in maintaining the predators as viable members of the aquatic community throughout seasonal variations.

The ratio of the number of bdellovibrios to total CFU on the shell was generally observed to increase with the time of submersion. This was due primarily to the continuous increase in the numbers of bdellovibrios over the 120-h period of the experiment and the small increase in the number of CFU after 24 h. Interestingly, the PFU/CFU ratio on glass and plastic did not increase, due to the continued elevation of CFU throughout the experimental period. However, differences in the ratios between all surfaces were not found to be significant.

A host of other environmental parameters may influence the colonization of bdellovibrios on surfaces. These factors may include surface charge (4), species of heterotrophic bacteria colonizing the surface, chemical conditioning, and sufficient biofilm accumulation to anchor bdellovibrios so that the number accumulating and persisting at the surface exceeds the number lost or washed off. Clearly, the factors which influence bdellovibrio association with surfaces are neither singular or simple but, rather, multiple and complex. To better understand the ecology and behavior of bdellovibrios in the aquatic environment, further study of the physical, chemical, and biological variables on their association with surfaces is essential and warranted.

Much more information about the ecology of the predators, including their interactions with other organisms, may be obtained by the study of their behavior on surfaces. Also, the use of surfaces provides a method of studying these organisms in situ and under a wide range of environmental conditions. The time during which the populations are allowed to develop on the surfaces is controlled by the investigator, since individual test surfaces can be retrieved at any time. Surfaces can be transported into the laboratory and studied by any number of means including culture, direct microscopic observation by specific labelling techniques, and molecular techniques. In laboratory culture vessels, bdellovibrios have been observed to adhere to certain surfaces (10), thus expanding the study of these organisms under more controlled conditions.

Since the discovery of the bdellovibrios, many questions have been asked about the role of these predatory bacteria in nature and their impact on populations of their prey. Attempts to design studies to resolve these questions and to understand more about the ecology of the predators have been limited, typically, to the study of the organisms suspended in the water column, in laboratory liquid cultures, or in sediments. The lack of substantial and meaningful data from study of the water column led Shilo and Bruff (14) to comment that perhaps investigators have been examining the wrong habitats. The use of surfaces to study the bdellovibrios has largely been neglected until recently (29). The association of bdellovibrios with surfaces appears to be an important facet in the ecology of these predatory bacteria.

In this study, bdellovibrios were recovered in large enough numbers from surfaces to influence bacterial populations. The accumulation of bdellovibrios at surfaces over a wide temperature and salinity range and on occasions when the numbers were below the limits of detection in the water column suggests that surfaces are a conducive and perhaps preferred habitat for these organisms. The use of surfaces to study bdellovibrios offers potential for uncovering much more information about these organisms and will perhaps lead to an understanding of their role in nature.

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