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Distribution of the Luminous Bacterium *Beneckea harveyi* in a Semitropical Estuarine Environment

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Bioluminescent bacteria were found in the water column, sediment, shrimp, and gastrointestinal tract of marine fishes from the semitropical estuarine environment of the East Lagoon, Galveston Island, Tex. Populations in the water column decreased during cold weather while sedimentary populations persisted. The highest percentages of luminous organisms were isolated from the gastrointestinal tract of marine fishes, where they persisted during 5 days of starvation. The presence of chitin temporarily increased intestinal populations. All isolates were *Beneckea harveyi*, whose natural habitat appears to be the gut of fishes and whose free-living reservoir appears to be marine sediments.

Luminescent bacteria are found saprophytically and parasitically on marine animals and live symbiotically in specialized organs in certain marine fish and cephalopods (2). Although the biochemistry, physiology and, most recently, the taxonomy of luminous bacteria have been studied extensively (9, 19, 22, 23, 29), the ecology of these organisms has been relatively neglected.

Recent studies in classification have concluded that there are three major groups of luminous bacteria (6, 13, 22). The first group contains *Photobacterium fischeri*. *Photobacterium leiognathi* and *Photobacterium phosphoreum* comprise the second group of luminous bacteria (3, 12, 22), and the third group contains *Beneckea* (*Lucibacterium*) *harveyi*, *Beneckea* (*Photobacterium*) *splendida*, and *Vibrio cholerae* biotype *albensis* (3, 12, 22).

The two *Photobacterium* groups contain members known to be symbiotically associated with higher organisms (4, 11, 12, 14, 22, 25, 26). The third group contains the three luminous *Beneckea* species which are not found as symbionts in luminous organs, but can be isolated directly from seawater, the surfaces of decaying fish and squid (9), or the surfaces of living marine animals (28, 22). Luminous members of the genus *Beneckea*, as well as *Photobacterium* sp., shed into the seawater from luminous organs are termed "free-living" bioluminescent bacteria (11).

A recent study has shown that there are seasonal changes of the free-living luminescent bacterial flora in surface waters off California. *P. fischeri* may be isolated from the water column throughout the year. However, *P. phosphoreum* was detected only in winter samples, whereas *B. harveyi* dominated the summer collections. A correlation between the ambient surface water temperature and the abundance of *B. harveyi* was observed (26).

The role of luminescence in symbiotically associated bacteria can be appreciated. The advantage of light emission to free-living and saprophytic luminous bacteria is less obvious. Free-living luminous bacteria can attach to the surface of marine crustaceans (e.g., penaeid shrimp) (7) and, if ingested by the proper fish, may establish residence in the fish gut (16). Thus, a mode of entry for bioluminescent bacteria into the gastrointestinal tract of a fish can be postulated. The goal of this research was to examine the distribution of free-living luminous bacteria in an estuarine environment in hopes of understanding the ecological role of bioluminescence in these bacteria.

METHODS AND MATERIALS

Sampling. (i) Habitat description. The seasonal abundance of luminous bacteria was followed in the East Lagoon. The lagoon, which is located on the eastern end of Galveston Island, Tex., is a narrow body of water, 1.8 km long and 71 m wide at the narrowest point. It empties into Galveston Bay about 0.4 km north of the Gulf of Mexico. The head of the lagoon has a depth of about 4.4 m and receives the greatest amount of drainage from the surrounding marsh. The mouth of the lagoon is 2.5 m deep and subject to greater tidal action (30). The lagoon supports a wide variety of small fish and crustaceans.

(ii) Sampling apparatus and procedure. Water-sediment samples, shrimp, and fish were collected from the East Lagoon for 12 months (June 1977 to June 1978). Water samples were collected in sterilized milk dilution bottles, capped, and returned to the laboratory within 1 h for processing.

Sediment samples were collected in a polypropylene centrifuge tube with a hole drilled in the bottom. The

presterilized tube was forced into the sediment, and a rubber stopper was inserted in the hole. The sample was immediately transferred to a sterile glass jar (250 ml) and returned to the laboratory for plating.

Shrimp and fish were collected in a 10-foot (ca. 3.05 m) otter trawl. The specimens were transferred to sterile wide-mouth jars (250 ml) and placed on ice. They were aseptically dissected upon return to the laboratory, usually within 1 to 3 h. Fish collected from the East Lagoon for feeding experiments were transported live to the laboratory.

Water temperature, salinity, and dissolved oxygen were measured with each water-sediment sampling. Dissolved oxygen and surface water temperatures were taken with a YSI model dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio). Salinity measurements were made with a hand refractometer (American Optical, Buffalo, N.Y.).

Enumeration. The media used for enumeration and maintenance in this study were seawater yeast extract agar and modified seawater yeast extract agar. The media were prepared with either three-salts solution (6) or artificial seawater (22). The composition and preparation of these media has been described previously (5, 22). The spread plate method was the primary procedure for determining the number of bacteria in water, sediment, penaeid shrimp, or stomach or intestine of marine fish. Appropriate dilutions were made using sterile three-salts solution. All samples were plated in triplicate.

The bacteria associated with penaeid shrimp were determined by returning the shrimp to the laboratory where they were weighed in presterilized weighing boats and homogenized for 1 min in a cold Waring blender containing 100 ml of sterile three-salts solution. Portions of the suspension were plated in triplicate on seawater yeast extract agar.

Marine fish (*Cynoscion nothus, C. nebulosus, Micropogon undulatus, Pogonias cromis, Stellifer lanceolatus,* and *Leiostomas xanthurus*) (14, 21, 29) were returned to the lab for dissection. The fish were placed on sterile cloths, and the stomach and intestines of the fish were removed. Precautions were taken during the dissection to prevent contamination of the gut with flora from the fish surface by dipping each piece of dissection equipment in ethanol and flaming immediately before it was used. The dissected gut sections were weighed, homogenized in 100 ml of sterile three-salts solution and spread onto seawater yeast extract media. The plates were incubated at 25°C and were observed in a dark room at 24 and 48 h for luminescent colonies. The plates were counted after 48 h of incubation to determine total viable counts.

Taxonomic identification. Taxonomic identification of the luminous bacterial isolates was accomplished by using selected criteria established by Reichelt and Baumann (22). Specifically, the isolates were tested for growth on minimal media containing 1 of 10 carbohydrates (D-xylose, maltose, cellobiose, D-gluconate, D-glucuronate, mannitol, DL-lactate, pyruvate, acetate, and propionate). Two amino acids (L-proline and *d*-α-alanine) were also tested as sole carbon and energy sources. Reference strains (*B. harveyi, P. phosphoreum, P. leiognathi,* and *P. fischeri*) obtained from Kenneth Nealson of Scripps Institute of Oceanography, San Diego, Calif., were included for comparative purposes.

Starvation and feeding experiments. To examine the survival of luminous bacteria in the gut of starved fish, two experiments were designed with 35 and 50 fish, respectively. The fish were collected and returned live to the laboratory, where they were maintained in an aquarium without food for up to 12 days. Ten fish were sacrificed daily. The stomach and intestines were aseptically dissected, weighed, homogenized, and plated by techniques previously described.

Retention time was also determined for 60 fish fed a single dose of luminous bacteria under laboratory conditions. These fish, which had been starved for 2 weeks before the experiment, were fed one portion of shrimp inoculated with a nalidixic acid-resistant strain of *B. harveyi.* The nalidixic acid resistance marker was used to insure the identity of the luminous strain reisolated from the fish. Thirty minutes after feeding the fish, all uneaten portions were removed from the tank. In general, no nalidixic acid-resistant luminescent bacteria were found in the water column after removal of the shrimp. The fish were sacrificed daily for 6 days in one experiment and between 48 and 72 h in the second. Total viable counts and luminescent counts per gram (wet weight) of gut were made.

Additional fish (40) were divided into two groups which were fed equal portions of inoculated shrimp that either retained or had the exoskeleton removed. This experiment was an attempt to measure the effect of chitin on retention of bacteria in the fish gut.

In a third feeding experiment, the passage of luminous bacteria through the stomach into the intestine was measured. Five fish were sacrificed each hour for 12 h after one feeding of inoculated shrimp, and the contents of the stomach and intestine were examined.

RESULTS

Over a 12-month period, 27 water samples were collected from the East Lagoon, 15 of which (55.6%) contained bioluminescent bacteria. Total viable counts in the water column fluctuated from 3.4×10^2 to 3.6×10^4 bacteria per ml throughout the year, with an average of 5.2×10^3 (Table 1). The abundance of bioluminescent bacteria tended to vary with water temperature, with fewer than one per ml in water temperatures of 7°C (i.e., midwinter). The number of bioluminescent organisms gradually increased with increasing water temperatures (Table 2). The highest bioluminescent counts (38.3 per ml) were obtained during the summer months.

If the luminescent bacterial counts are separated on the basis of temperature, it can be observed that 12 of the 18 samples (66.7%) collected when temperatures were greater than 15°C contained luminescent bacteria. In contrast, only two of nine samples (22.9%) collected when temperatures were less than 15°C contained luminescent bacteria. The average water temperature of the East Lagoon was 23°C; there were only 3 months when the average water

TABLE 1. *Mean population of total and luminous bacteria occurring in materials from the East Lagoon, Galveston, Tex., during June 1977 to June 1978*

Sample	No. of samples examined	Total viable counts	Luminous counts	% Luminescence
Water (per ml)	27	5.2×10^3	4.9×10^0	0.09
Sediment (per g dry wt)	28	5.7×10^5	2.6×10^2	0.05
Shrimp (per g wet wt)	25	7.7×10^5	2.9×10^3	0.38
Stomach (per g wet wt)	21	4.8×10^6	2.1×10^4	0.44
Intestines (per g wet wt)	21	1.8×10^7	3.7×10^6	20.55

temperatures were <15°C.

Of the 28 sediment samples collected during this same period, 24 (85.7%) exhibited luminescent growth. Total viable counts for the sediment varied from 1.8×10^4 to 2.4×10^6 cells per g (dry weight) through the year. The total concentration of luminous cells, when detectable, varied from 1.6 cells to 1.3×10^3 cells per g (dry weight).

The counts of luminous bacteria per gram of sediment fluctuated greatly, and no correlation between luminous counts and water temperatures was observed. However, during the winter months (i.e., January and February), the incidence of luminous bacteria occurring in the sediment was greater than that for the water column.

Of the 25 shrimp examined, 20 (80%) contained luminescent bacteria. Total viable counts for the shrimp varied from 1.3×10^4 to 2.7×10^6 cells per g of shrimp. The highest number of bioluminescent bacteria in shrimp was obtained during the summer. Neither the percentage of luminescent bacteria nor the monthly counts per gram appear to be closely related to water temperatures. A higher percentage of luminous bacteria occurred in shrimp, on the average, than in the water column or the sediment (Table 1).

Bioluminescent bacterial counts varied from 1.3×10^2 to 4.2×10^5 cells per g of stomach tissue and 1.2×10^3 to 5.0×10^7 cells per g of intestine, when detectable.

There appeared to be no difference in the abundance of luminous bacteria in the gut of the various species of fish examined. Bacteria, when present in the stomach, were found in consistently lower percentages than in the intestines.

In microbiological work, the dilution factors and variations within samples tend to produce large statistical variances around the mean. A nonparametric test (i.e., Kruskal-Wallis test) was used in lieu of parametric analysis of variance (28). The field data show, with few exceptions, that the numbers and percentages of luminous bacteria increased in the following order: water column, sediment, shrimp, stomach, and intestines. Conclusions reached by the Kruskal-Wallis test indicated significant differences between the numbers of luminous bacteria, comparing water and sediment, sediment to shrimp, and shrimp to stomach at the 0.01 level of significance. There was also a significant difference between the percentage of luminous bacteria comparing sediment and shrimp and the percentage of luminous bacteria comparing shrimp and stomach. There was no significant difference between the numbers of luminous bacterial counts in the stomach and the luminous bacterial counts in the intestine. However, there was a significant difference between the percentage of luminous bacteria in the stomach and intestines (0.05 level of significance).

Taxonomic identification. The 79 luminous strains collected in the field study were identified following the criteria set forth by Reichelt and Baumann (22). Strains were characterized on the basis of nutritional versatility. Results from such tests indicate that all bioluminescent bacterial strains collected in the East Lagoon from June 1977 to June 1978 belong to the species *B. harveyi* (22) (Table 3). (*B. harveyi* is designated *Lucibacterium harveyi* in *Bergey's Manual* [6].) None of the *Photobacterium* spp. were isolated during this study.

TABLE 2. *Seasonal variation in total viable and luminous plate counts per milliliter with water temperature*

Sampling date	Luminous counts	Total viable counts	Water temp (°C)	% Luminescence
1977				
Jun	38.3	3.4×10^2	31.00	1.13
Jul	5.5	5.9×10^2	31.50	0.95
Aug	3.3	9.9×10^2	30.00	0.34
Sep	0	3.9×10^3	29.50	0
Oct	6.7	4.9×10^3	26.50	0.14
Dec	3.3	1.9×10^3	14.00	0.18
1978				
Jan	0	1.5×10^3	13.50	0
Feb	0	1.0×10^4	11.20	0
Mar	4.9	1.1×10^4	19.38	0.05
Apr	6.7	6.5×10^3	23.00	0.10
May	3.3	3.4×10^3	24.75	0.10

TABLE 3. *Comparison of taxonomic properties of test strains with B. harveyi*

Determination	Test strains[a]	*B. harveyi*[b]
Utilization of:[c]		
D-Xylose	−	−
Maltose	+	+
Cellobiose	+	+
D-Gluconate	+	+
D-Glucuronate	+	+
Mannitol	+	+
DL-Lactate	+	+
Pyruvate	+	+
Acetate	+	+
Propionate	+	+
L-Proline	V	+
D-Alanine	+	+
Production of extracellular enzymes:		
Lipase	+	+
Amylase	V	+
Gelatinase	+	+

[a] Results of 79 luminous test strains. +, 80% or more of the strains tested were positive; −, 5% or less of the strains tested were positive; V, between 5 and 80% of the strains tested were positive.

[b] Reichelt et al. (24).

[c] As sole source of carbon and energy.

Laboratory data. The results from 35 starved fish indicate a decrease in the number of bioluminescent bacteria per gram of intestine (Fig. 1) and in the percentage of fish retaining luminous bacteria as the period of starvation increased. Short-term (5 day) comparison of the percentages of 50 starved fish showed a decrease of luminous bacteria in both the stomach and intestine, with a faster decrease occurring in the stomach (Fig. 2).

Sixty starved fish were fed shrimp inoculated with *B. harveyi*. The luminous bacterial counts on the inoculated shrimp pieces ranged from 1.7×10^6 to 4.1×10^{11} cells per g, with no difference between pieces with and without exoskeleton. The result of the daily average luminescent counts per gram for the stomach and intestines of fish fed the shrimp can be seen in Fig. 3. As in the starvation experiment, there was an overall decrease in the numbers of bioluminescent bacteria present in the intestine over time. A slight difference occurred between fish with and without chitin in their food during the first 3 days of the experiment. The numbers of luminous bacteria in the stomach declined at a faster rate than in the intestine. As in the starvation experiment, there was a sudden increase in the numbers of luminous bacteria at 5 days. All uneaten remnants of food were removed from the tank; therefore, no obvious source of gross contamination was present.

As a result of the first feeding experiment, an experiment was designed to examine numbers of bioluminescent bacteria in the fish gut during a short period of time (<3 days) when a source of chitin was either present or absent. The luminescent bacterial counts were 2.0×10^3 and 8.2×10^3 cells per g of stomach for fish fed food with or without chitin, respectively. In the intestines of those fish receiving shrimp with a chitin source, the counts were 1.7×10^5 per g. The counts in the intestines of those fish lacking a chitin source were significantly lower at 6.4×10^4 per g ($P < 0.01$, Kruskal-Wallis).

In the 12-h feeding experiment, plates were scored as to whether or not they showed luminescent bacteria. Bioluminescent bacteria were immediately detectable in both the stomach and intestines after the 30-min feeding time. Luminescent bacteria continued to be present in the gastrointestinal tract for the entire time period.

DISCUSSION

In this study luminous bacteria were found to occur throughout the year in the East Lagoon, near Galveston, Tex. These bacteria were found to associate with penaeid shrimp and the gastrointestinal tracts of marine fish (i.e., *Sciaenidae*) and to occur free-living in the water column and sediment.

Seasonal variation of species composition of

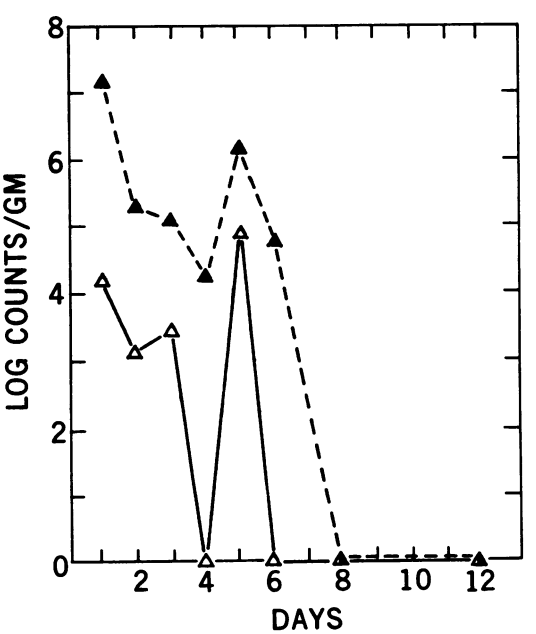

FIG. 1. *Average luminescent counts per gram of intestines (▲) and stomach (△) in starved fish over time.*

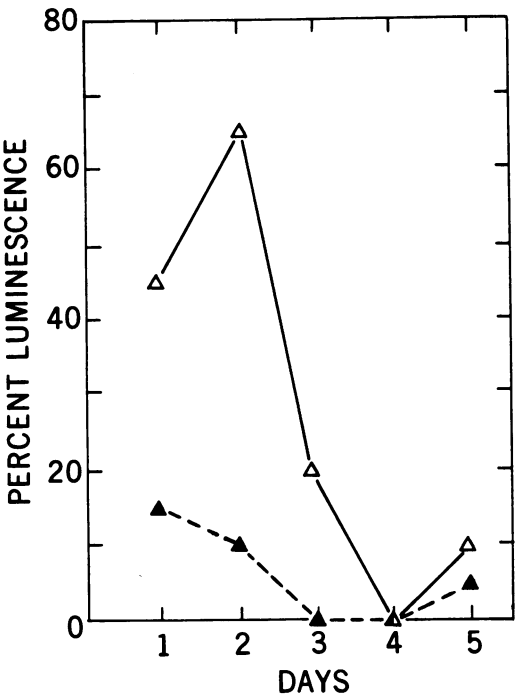

FIG. 2. *Percentage of fish with luminous bacteria present in the intestine (▲) and stomach (△) during a 5-day period. Each dot = 10 fish.*

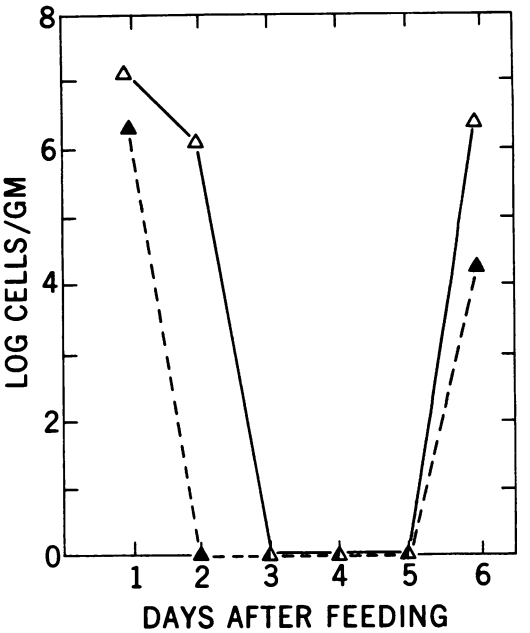

FIG. 3. *Luminous bacterial counts per gram (wet weight) of intestine compared over time in fish fed shrimp with (△) or without (▲) exoskeleton. Each dot = 5 fish.*

the bioluminescent bacterial population off the California coast has been reported with *P. fischeri* dominant in fall and winter samples and *B. harveyi* dominant in the summer samples (28). All bioluminescent bacteria isolated in this study were identified as *B. harveyi*. None of the *Photobacterium* groups were detected in the East Lagoon. The ability of *Beneckea* to grow at higher temperatures than *Photobacterium* (12) probably accounts for the dominance of *Beneckea* among luminous bacteria in the study area. Furthermore, fish containing endosymbiotic *Photobacterium* in the Gulf of Mexico are generally found in water at depths of 200 to 2,000 m (18). These depths are not found for several hundred miles off Galveston.

The free-living *B. harveyi* strains observed in this study were found to have a pattern of seasonality which included relatively constant numbers in the sediment and increasing numbers in the water column as the water temperature rises. This pattern is identical to that reported for *Vibrio parahaemolyticus* in the Chesapeake Bay (15). Indeed, *B. harveyi* showed similar, if not identical, physiological, taxonomic, and ecological characteristics to the marine *Vibrio* sp. (6, 15).

An inducible enzyme system in luminous bacteria permits the bacteria to maintain either a luminous or nonluminous existence. In a dilute environment, such as the ocean, free-living luminous bacteria are unable to synthesize luciferase or emit light (11). The highest number of luminous bacteria found in this study was associated with marine animals, including penaeid shrimp and fish gut, where concentrated numbers could allow for light emission (i.e., as in a lesion or fish feces). Such association, followed by ingestion by potential predators, would enable luminous bacteria to enter (or reenter) the gastrointestinal tract of marine fish. The acidic environment of the stomach is presumably not suitable for growth and reproduction, but *B. harveyi* was able to survive and grow in these conditions. The pH of the intestines (1) and nutrients (e.g., chitin) available in the gut of well-fed fish would be more advantageous for the propagation of luminous bacteria and would result in higher numbers as found in this study. These data support the hypothesis that luminous bacteria are primarily enteric symbionts that may also live in other niches before reentry into the fish gut.

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