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Distribution and Identification of Luminous Bacteria from the Sargasso Sea

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Vibrio fischeri and *Lucibacterium harveyi* constituted 75 of the 83 luminous bacteria isolated from Sargasso Sea surface waters. *Photobacterium leiognathi* and *Photobacterium phosphoreum* constituted the remainder of the isolates. Luminescent bacteria were recovered at concentrations of 1 to 63 cells per 100 ml from water samples collected at depths of 160 to 320 m. Two water samples collected at the thermocline yielded larger numbers of viable, aerobic heterotrophic and luminous bacteria. Luminescent bacteria were not recovered from surface microlayer samples. The species distribution of the luminous bacteria reflected previously recognized growth patterns; i.e., *L. harveyi* and *V. fischeri* were predominant in the upper, warm waters (only one isolate of *P. phosphoreum* was obtained from surface tropical waters).

Marine luminous bacteria comprise gram-negative motile rods, the single, most unique trait of which is the emission of light. Beijerinck in 1889 (2a) recognized the unique nature of bioluminescence and proposed that all light-emitting bacteria be placed into a single genus, *Photobacterium*. Taxonomic studies have since revealed new luminous bacterial species possessing a large number of phenotypic characters common to members of the *Enterobacteriaceae* and *Vibrionaceae*. As described in *Bergey's Manual of Determinative Bacteriology* (3) and by Hendrie et al. (6), there are three genera and five species of luminous bacteria, *Vibrio cholerae* biotype *albensis, Vibrio fischeri, Lucibacterium harveyi, Photobacterium phosphoreum,* and *Photobacterium mandapamensis*. Based on results of their recent studies, Baumann and coworkers (1, 2) and Reichelt and co-workers (9-11) proposed major revisions in the assignment of genera and designation of species of luminescent bacteria, based primarily on deoxyribonucleic acid (DNA) base composition, in vitro DNA/DNA and DNA/ribosomal ribonucleic acid hybridization, nutritional versatility, and mode of flagellation. Whereas specific epithets for the luminescent bacteria have not been agreed upon, species assignments have been made on the basis of nutritional, enzymatic, and growth characteristics.

The luminous bacteria are widely distributed in the world oceans in one or more states of existence. Nealson (8), Ruby and Morin (12, 13), and Ruby and Nealson (14) have documented the symbiotic relationships of luminous bacteria with marine fishes. It seems well established that all such symbioses are species specific (12, 14); e.g., *P. phosphoreum* is the only luminous symbiont of three species of the bathyal fish family *Macrouridae*, and *Photobacterium fischeri* is specific for the monocentrid fish *Monocentris japonica*. In addition, individual light organs are composed of only one luminous bacterial species, and *Lucibacterium* (*Beneckea*) has never been identified as a light-organ symbiont. All species appear to be potential gut symbionts, saprophytes or free-living forms, and all, except *Photobacterium leiognathi*, have been observed in parasitic association.

The distribution and species composition of marine luminous bacteria have been largely ignored until recently. Pioneering work by Beijerinck in the North Sea and recent work by Yetinson and Shilo (19) in the Mediterranean Sea and the Gulf of Elat suggest a seasonal variation in the species composition of free-living luminous bacteria caused by fluctuation of several abiotic factors, such as temperature, salinity, sunlight, and nutrients. Ruby and Nealson (14) described the seasonal variation of *P. fischeri* and *Beneckea harveyi* in California coastal surface waters and enumerated populations of the species over a 2-year period. We report here the spatial distribution of populations of luminous bacterial species in the upper water layer (160 to 320 m) and surface microlayer of the Sargasso Sea along a cruise track line from Miami, Fla., to Recife, Brazil.

MATERIALS AND METHODS

Locations of the sampling sites are given in Fig. 1. Microlayer samples were collected at eight stations, using a glass plate sampler (4) suspended from the bow of the ship, which was traveling at a speed of 1

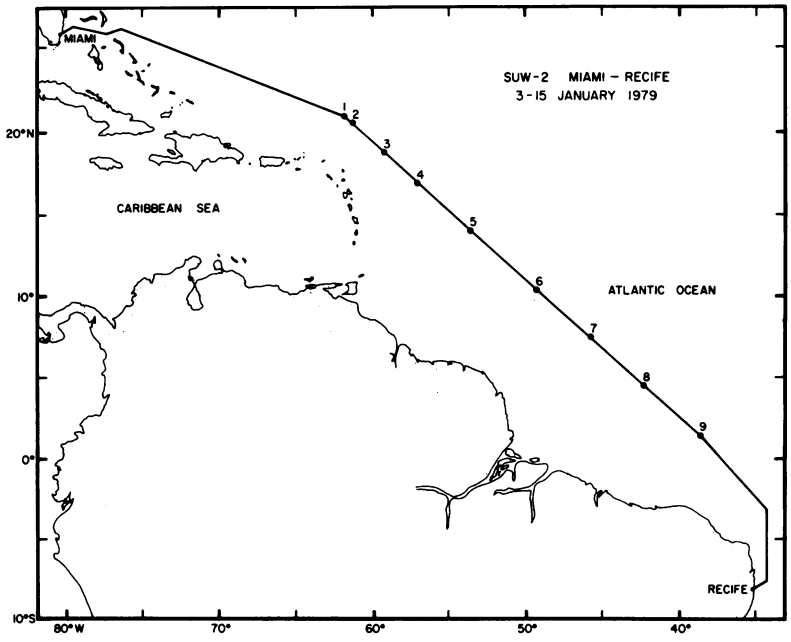

FIG. 1. *Station locations included in the R/V Researcher cruise SUW-2.*

knot. Subsurface seawater samples were collected aseptically, using a Niskin bag sampler, and the samples were processed immediately after recovery.

Total, viable, aerobic heterotrophs and luminescent bacteria in the Sargasso Sea water samples were collected by filtration, using 0.2-μm membrane filters (Millipore Corp., Bedford, Mass.). The filters were placed on modified MSWYE agar (16) made up with a 24‰ marine salts solution. The plates were incubated for 2 to 3 days at 25°C (in situ water temperature) in the dark. Luminescent bacteria, after incubation for 24 h at 25°C, were identified by luminescence under low-level illumination and were picked, using sterile toothpicks, to fresh MSWYE agar plates and incubated at 25°C. Further purification, testing, and storage of luminescent strains was done with SWC agar (8) buffered with 1 g of calcium carbonate per liter.

Total, direct counts of bacteria were made by filtering aliquots of seawater samples, using irgalan black-stained, 0.2-μm, Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.), and staining the filters with 0.01% acridine orange for 4 min (7). The filters were viewed by epifluorescence microscopy, and 10 to 20 fields per sample were counted to determine total numbers of bacteria.

Quadruplicate replicates of duplicate subsamples of each seawater sample were used in both the plate count and the direct count of bacteria to minimize sample variance arising from patchiness, sample processing, etc.

Identification of the luminous isolates followed a modification of the scheme of Reichelt and Baumann (9). For purposes of communication, the nomenclature designations for *Lucibacterium*, *Vibrio*, and *Photobacterium* have been included. The diagnostic characters proposed by Reichelt and Baumann (9) for species differentiation were used, with *P. leiognathi*, not presently recognized in *Bergey's Manual* (8th edition), also included among the species of luminous bacteria (10). The diagnostic traits used to identify luminous isolates obtained in this study included: Gram reaction, presence and mode of insertion of flagella, oxidase and catalase reactions, growth at 4 and 40°C, production of amylase, lipase, and gelatinase, production of gas from glucose, and utilization of mannitol, lactate, pyruvate, acetate, and D-α-alanine as sole sources of carbon and energy. Test media were incubated at 25°C, and all reactions were recorded within the period of time recommended by Nealson (8).

Results of the total plate count, direct count (acridine orange-stained samples), and luminous bacterial counts were processed by log transformation and subjected to correlation analysis, using the Biomedical Package statistical computer package.

RESULTS AND DISCUSSION

The total number of luminous bacteria varied from 1 to 63 cells per 100 ml, and the population of luminous bacteria constituted 0.6 to 7.6% ($\bar{x}$ = 2.5%) of the total, viable, aerobic heterotrophic bacteria enumerated by the plate counts. Ruby and Nealson (14) and Nealson (8) reported an average of 1 to 7.5 luminous bacteria per ml in the nearshore waters of California. Probably, the 100-fold difference observed in the number of luminous bacteria is indicative of a higher rate of production and increased available nutrients in neritic waters, as opposed to midocean

waters. The maximum number of luminous bacteria was isolated from water samples collected at a depth of 200 m, usually coinciding with the thermocline, where the number of luminous bacteria ranged from 14 to 63 cells per 100 ml (Table 1). Total counts of viable, aerobic, heterotrophic bacteria averaged 0.8 to 7.6 cells per ml, whereas direct counts indicated populations of 4 to 5 logs higher than the plate counts. The majority of cells stained by acridine orange and observed by epifluorescence microscopy were very short rods (0.3 to 0.6 μm) or cocci which yielded a green or white-green fluorescence. The extremely low concentration of suspended particulates in the water samples resulted in little nonspecific staining and no interference with counting. The dramatic difference observed in the numbers demonstrates the inefficiency of the plate count method observed for enumerating bacterial populations and reflects the oligotrophic state of the Sargasso Sea, where production is one-half, or less, that of the Boreal ocean (17).

At the 5% level of significance (df = 11; critical $r = 0.55$), neither acridine orange direct counts ($r = 0.49$) or total plate counts ($r = 0.33$) were observed to be correlated with numbers of luminous bacteria, suggesting that the distribution of luminous bacteria at depths of 160 to 320 m in the Sargasso Sea is nearly independent of total bacterial numbers and that the role of luminous bacteria is one of symbionts of fish rather than one of free-living organisms.

Surface microlayer samples, collected at six stations during the day and two of the stations at night in the Sargasso Sea, failed to yield any luminous bacteria. Hence, migration of planktonic luminous bacteria to the surface does not occur. Factors other than temperature, such as algal production of antibiotics, bacterial interactions (18), and solar irradiation (19), very likely act to prevent accumulation of luminescent bacteria at the surface microlayer.

Of the 83 luminous bacterial isolates obtained in pure culture, 54 were identified as *Lucibacterium* (*Beneckea*) *harveyi*, 21 were *Vibrio* (*Photobacterium*) *fischeri*, and 7 were *P. leiognathi*. Only one strain of *P. phosphoreum* was recovered (Table 2). These species constituted 65, 25, 8, and 1%, respectively, of the total number of luminous bacteria isolated in this study. Two species, *L. harveyi* and *V. fischeri*, accounted for 90% of the isolates. The distribution of *L. harveyi* and *V. fischeri* was approximately the same at all stations, and *P. leiognathi* was found only at station 6, where biological productivity and nutrient concentrations were highest of all the stations examined in this study. The single isolation of *P. phosphoreum* was from a water sample collected at a depth of 160 m, where the temperature of the water was 16°C.

The species distribution of luminous bacteria observed in this study is similar to that reported by Ruby and Nealson (15) for California coastal waters, where *L. harveyi* and *V. fischeri* accounted for 99% of all luminous species and *L. harveyi* composed 60 to 70% of the total luminous species during the summer months, when water temperatures averaged ca. 20°C. They

TABLE 1. *Physical, chemical, and microbiological parameters for stations in the Sargasso Sea*

Station	Depth (m)	Temp (°C)	Salinity (‰)	Total no. of bacteria[a]		Luminescent bacteria[d] (no./ml)	% Luminescent bacteria[e]
				Plate count[b]	AODC[c]		
1	320	18.2	36.5	0.8	3.75×10^4	0.01	1.0
2	160	23.0	37.0	1.4	4.12×10^4	0.12	2.1
4	160	22.6	35.0	7.6	5.14×10^4	0.09	2.9
	240	19.3	36.7	1.4	1.24×10^5	0.28	7.6
	280	17.9	36.5	1.0	6.50×10^4	0.02	2.2
5	160	21.6	35.0	1.5	1.16×10^5	0.02	0.7
	200	19.7	36.9	1.8	2.06×10^5	0.63	7.1
	240	18.1	36.6	1.1	6.35×10^4	0.06	1.4
	280	16.6	36.4	1.7	ND[f]	0.06	1.1
6	160	20.7	35.0	1.7	ND	0.08	0.9
	200	18.4	36.7	1.7	4.23×10^4	0.14	1.6
	240	17.4	36.4	1.2	ND	0.12	1.9
	280	14.9	36.0	1.4	ND	0.12	1.7

[a] Total viable count of aerobic, heterotrophic bacteria per milliliter of seawater.

[b] Grand mean of quadruplicate plate counts of duplicate seawater subsamples, expressed as the total number of viable, aerobic, heterotrophic bacteria (colony-forming units) per milliliter.

[c] Direct count of acridine orange-stained seawater samples, expressed as the number of fluorescent cells per milliliter.

[d] Enumerated from plate counts.

[e] Calculated as percentage of the plate count.

[f] ND, Not determined.

TABLE 2. *Distribution of luminous strains, by depth, for Sargasso Sea stations*

Station	Depth (m)	No. of luminous strains[a]		
		L. harveyi	*V. fischeri*	*P. leiognathi*
2	160	2	5	0
4	160	3	0	0
	240	1	0	0
	280	1	0	0
5	160	2	0	0
	200	17	9	0
	240	3	0	0
	280	2	2	0
6	160	6	0	0
	200	3	4	1
	240	8	1	3
	280	8	0	3

[a] One isolate of *P. phosphoreum* was recovered from station 4 at a depth of 160 m. No luminous bacteria were recovered from surface microlayer samples collected at eight stations.

also observed that *P. phosphoreum* occurred only in water samples collected during the winter months, when the temperature reached 13 to 14°C, but they did not detect *P. leiognathi* at any time of the year. Ruby and co-workers (13, 15) attribute the species seasonal distribution pattern observed in their studies to the differential effect of temperature wherein the growth rate of *L. harveyi* is maximal at temperatures above 18°C and the doubling time of *V. fischeri* is three- to fourfold faster than that of *L. harveyi* at 7°C. Thus, *L. harveyi* and *V. fischeri* would be expected to proliferate in tropical surface waters, such as the Sargasso Sea, but the presence of *P. leiognathi* in significant numbers at station 6 indicates the role of some other factor, e.g., shedding from a specific species of marine fish, which may not occur in California nearshore waters. It was impossible to determine whether these isolates were "free living" (5) or transient symbionts surviving in a new ecological niche. However, recovery of these bacteria in significant numbers at only one station may indicate a significant source of inoculum or peculiar ecological factor(s) unique to the geographical area examined in this study.

Yetinson and Shilo (19) reported that *Photobacterium* spp. are dominant under oligotrophic conditions, which was not the case in the Sargasso Sea, where ca. 34% of the species were *Vibrio* (*Photobacterium*) spp. The lack of dominance of *L. harveyi* (65%) or *V. fischeri* (25%) in the Sargasso Sea surface waters was in contrast to the rather special environments sampled by Yetinson and Shilo, e.g., the Bitter Lakes and the Gulf of Elat, where *Lucibacterium* (*Beneckea*) dominates in shallow water and *Photobacterium* dominates in deep waters.

Phenotypic characters expressed by *P. leiognathi* and *V. fischeri* are listed in Table 3. Some characteristics may reflect the environment from which the strains were isolated. For instance, 71% of the *P. leiognathi* strains grew at 4°C and none grew at 40°C, suggesting bathyal origin of the strains. Ninety-five percent of the *V. fischeri* strains demonstrated gelatinase activity, whereas Reichelt and Baumann (9) reported only 8% of their strains to be gelatinase positive. Sieburth (18), in his study on the biochemical activity of marine bacteria, found 90 to 100% of the strains to be gelatinase positive, and these represented the dominant bacterial group in the microflora of water samples collected at 103- to 220-m depth in the Atlantic Ocean. *V. fischeri* strains from the Sargasso Sea may possess gelatinase activity which permits utilization of gelatin-like substrates present in these waters, whereas the strains of Reichelt and Baumann (9) may have originated from waters not containing such substrates. In addition, 81% of the *V. fischeri* isolates utilized pyruvate, whereas none of the strains described by Reichelt and Baumann (9) did so. Approximately 80% of the strains described by Ruby and Nealson (15) utilized pyruvate as a sole carbon and energy source.

In summary, from the results of this study, it is concluded that the numbers and species of luminous bacteria found in the Sargasso Sea reflect the stable, warm, oligotrophic conditions in the surface waters serving as the aquatic habitat of these forms.

TABLE 3. *Characteristics of luminescent bacterial strains isolated*[a]

Characteristic	*L. harveyi* ($n = 54$)	*P. leiognathi* ($n = 21$)	*V. fischeri* ($n = 7$)
Oxidase	+	+	+
Catalase	+	+	+
Amylase	+	−	−
Lipase	+ (91%)	−	+ (81%)
Gelatinase	+ (92%)	−	+ (95%)
Mannitol	+	−	+
Lactate	+	+	−
Pyruvate	+	+	+
Acetate	+	−	−
D-α-Alanine	+	+	−
D-Glucose	Acid/no gas	Acid/no gas	Acid/no gas
Growth at:			
40°C	− (59%)	−	−
4°C	− (96%)	+ (71%)	−

[a] All strains were gram negative. *L. harveyi* and *P. leiognathi* were motile by means of a single polar flagellum. *V. fischeri* possessed a tuft of polar flagella. Approximately 24% of the *L. harveyi* isolates and 29% of the *P. leiognathi* strains produced dark (nonluminescent) variants after three successive transfers.

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