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Distribution of Ultramicrobacteria in a Gulf Coast Estuary and Induction of Ultramicrobacteria

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Abstract. The abundance of ultramicrobacteria (i.e., bacteria that pass through a 0.2 μm filter) in a subtropical Alabama estuary was determined during a 1-year period. Although phenotypic and molecular characterization indicated that the population of ultramicrobacteria was dominated by *Vibrio* species, species of *Listonella* and *Pseudomonas* were also abundant. Vibrios occurred with the greatest frequency in waters whose salinities were less than 14‰, and were the most abundant species of the total ultramicrobacterial population year-round, while *Pseudomonas* species were absent or considerably reduced during the winter months. The total number of ultramicrobacteria showed an inverse relationship to total heterotrophic bacteria as measured by colony-forming units (CFU)/ml and to water quality as measured by several parameters. Analysis by generic composition indicated that both salinity and temperature significantly affected the distribution of these organisms. Laboratory studies revealed that strains of vibrios under starvation in both static and continuous-flow microcosms could be induced to form cells that passed through 0.2 and/or 0.4 μm filters. Cells exposed to low nutrients became very small; some grew on both oligotrophic (5.5 mg carbon/liter) and eutrophic (5.5 g carbon/liter) media; and some few cells grew only on oligotrophic media. By passing selected vibrio strains on progressively diluted nutrient media, cells were also obtained that were small, that passed through 0.4 μm filters, and that could grow in oligotrophic media. These results suggest that ultramicrobacteria in estuaries (at least some portion of the population) may be nutrient-starved or low nutrient-induced forms of certain heterotrophic, eutrophic, autochthonous, estuarine bacteria.

Introduction

The presence of extremely small microorganisms in aquatic environments is well documented [31, 36, 49, 52]. The diversity of the aquatic environments from which these very minute bacteria have been isolated, i.e., arctic waters [36], temperate bays [52], the deep sea [49], and a subtropical Gulf Coast estuary

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[31], suggests that these bacteria may be ubiquitous in water bodies. Furthermore, most of the nucleic acid in ocean waters has been shown to be associated with these small bacteria as the fraction of filtered waters that passed through 0.2–1 μm filters contained the highest concentration of DNA [42].

Although there are a variety of terms used to describe these bacteria, including "minibacteria" [52] (not to be confused with DNA deficient, laboratory-induced "mini cells" [13]), "ultramicrobacteria" [32, 51], and "microvibrios" [16], the feature common to all these descriptions is that the microorganisms are considerably smaller than those routinely isolated from aquatic environments using standard procedures. While the nature of these organisms has remained somewhat obscure due to inadequate methods for their isolation, enumeration, and characterization, estuarine ultramicrobacteria were isolated and characterized using membrane filters and low nutrient media [31].

Several hypotheses have been suggested as to the nature of these microorganisms [31, 32, 37, 51, 52]. They may be bacteria that, while morphologically smaller than other strains, represent organisms of identifiable taxa (i.e., they are not taxonomically "new" species). They may be organisms whose size and morphology are the result of responses to nutrient deprivation. Reduction in cell volume as a result of carbon and/or nitrogen limitation has been observed for a number of bacteria including *Escherichia coli* [6], *Salmonella* spp. [45], *Vibrio cholerae* [3], and a large number of marine and freshwater bacteria [2, 12, 23, 27, 28, 37, 39, 40, 41, 44]. Such size reduction in marine bacteria has been characterized as reductive division [39], fragmentation [28], and generally, as dwarfing [23].

In an attempt to understand the relationships among certain environmental parameters and the occurrence of these small bacteria, the distribution and generic composition of bacteria that passed through a 0.2 μm filter (referred to as 0.2 μm filterable bacteria) were examined in a subtropical estuary—Perdido Bay, Alabama—during a 1-year period. Furthermore, an attempt to understand the relationships between nutrient levels, bacterial size, and nutrient requirements was made using strains of selected vibrios that were nutrient stressed to determine if ultramicrobacterial cells could be induced in the laboratory. This report describes the results of those studies.

Materials and Methods

Distribution Studies

Sampling Sites. Water samples were collected from five sites in Perdido Bay, Alabama (Fig. 1). Samples sites were selected on the basis of salinity and apparent extent of human impact. Table 1 describes selected water quality parameters of the sites. pH ranges have been previously reported [32]. Site A, at the mouth of the bay, represented the highest salinities, good water quality, and greatest potential for exchange with Gulf waters. Site B, located on an extensive shallows, was characterized by moderate salinites, was least influenced by human activities, and exhibited the best water quality. Site C, with the lowest salinities, was located in a heavily populated cul-de-sac formed by the ingress of the Perdido River. Waters at this site were heavily influenced by urban development, boating, and waste disposal. Site D, located closest to the mouth of the Perdido River, also exhibited poor water quality due, in part, to petroleum spillage from boats associated

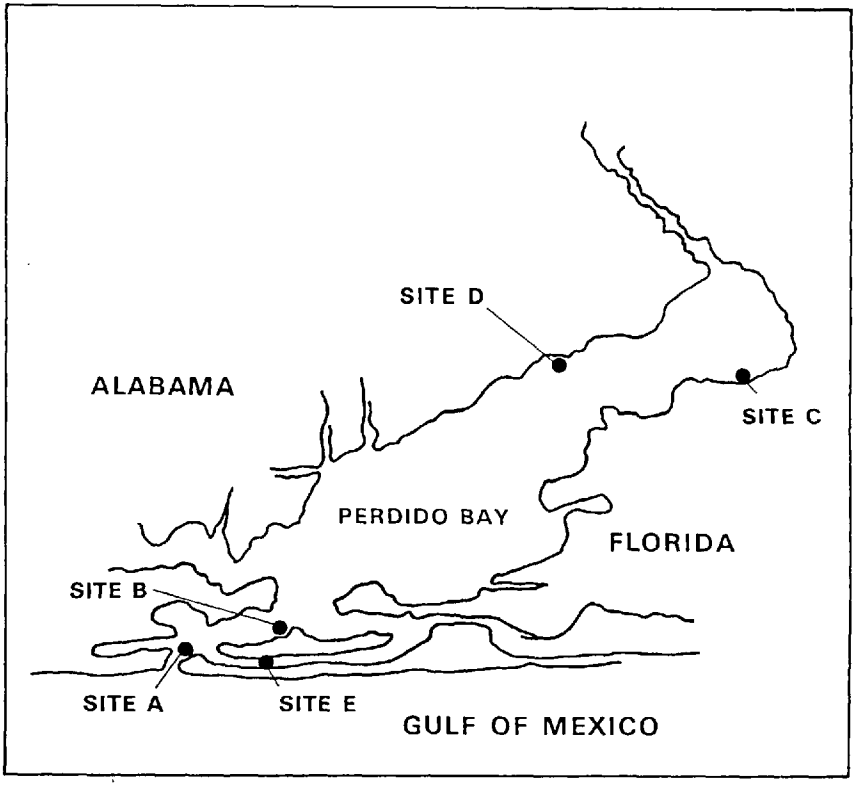

Fig. 1. Location of sampling sites in Perdido Bay, Alabama.

with several adjacent marinas, while site E was subject to moderate impact from swimming and pleasure boating, and had a high potential for exchange with Gulf waters.

Sample Collection. Water samples were collected every other month from approximately 30 cm below the surface, in clean autoclave-sterilized glass bottles. Sample bottles were capped aseptically, placed in foam coolers for transport to the laboratory, and analyzed within 4 hours. In order to minimize possible effects of temperature stress, samples were maintained at temperatures approximating those of the water from which they were collected. Salinity was determined at the time of sampling using a temperature-compensated TS meter (American Optical, Keene, New Hampshire). Water temperature was measured by complete submersion of a laboratory thermometer (Fisher Scientific Products, Inc., Fairlawn, New Jersey) into site water. pH was measured using a field pH meter (Sargent-Welch Scientific Co., Birmingham, Alabama). Total organic carbon, BOD, organic nitrogen, total Kjeldahl-nitrogen, ammonium-nitrogen, nitrite plus nitrate-nitrogen, and total-phosphorus were determined using standard methods [1] by the Department of Environmental Regulations (Pensacola, Florida).

Enumeration and Isolation of Ultramicrobacteria. Techniques used for the enumeration of the 0.2 μm filterable bacteria were modifications of those previously described for isolating ultramicrobacteria [31]. Using the three-tube series Most Probable Number (MPN) procedure, water samples were filtered through 0.2 μm pore size Nuclepore polycarbonate filters (Nuclepore Corp., Pleasanton, California), diluted in marine salts solution (Instant Ocean, Aquarium Systems, Mentor, Ohio), and inoculated into dilute Lib-X-broth [40]. The tubes were incubated at 20 and 35°C for 21 days.

Isolation and enumeration consisted of streaking each tube onto a low-nutrient solid medium, brain heart infusion estuarine salt (BHES), as described previously [31]. MPNs were then calculated on the basis of growth on BHES.

Identification of Bacterial Isolates. All isolates were subjected to a series of biochemical and physical tests using media diluted ⅛ of the recommended strength with marine salts solution and adjusted to a final salinity of 15‰, as previously described [31]. Decarboxylase Base Moeller (Difco Laboratories, Detroit, Michigan) was used in carbohydrate utilization assays. Lactose fermentation was determined by the method of Hugh and Leifson [22]. Resistance to the vibriostatic agent, 0/129 (2, 4-diamino-6, 7-diisopropyl pteridine phosphate, Sigma Chemicals, St. Louis, MO) was assayed using sterile 5 mm discs of Whatman 1 filter paper (W. & R. Balston, London, UK) impregnated with 10 and 150 mg of the vibriostat in acetone. The discs were allowed to dry completely before use. VP and salt requirements were determined as previously described [14]. Cytochrome oxidase and B-galactosidase activities were assayed using N, N, N′, N′-tetra-methyl-p-phenylenediamine (Sigma) and o-nitrophenyl-galactropyranoside (ONPG, Sigma), respectively. The string test was performed [48]. Assignments of taxa were based on the identification rationale of Furniss et al. [14] and other identification characteristics [4, 5, 7, 8, 10, 11, 18, 19, 24–26, 38, 46–48, 50].

DNA base ratios, i.e., GC mol%, (G+C/A+T+G+C) were determined using the polyacrylamide-agarose gel technique of MacDonell et al. [33] modified from Zeiger et al. [53] and Gilpin and Dale [15]. Cells were conditioned to grow on 2 g/ml trypticase [31], cultured in Marine Broth 2216 (Difco) for 18 hours at room temperature (ca. 22°C) on rotary shakers (150 rev/min) and harvested by centrifugation (2,000 × *g*; 30 min). The DNA was extracted by the method of Marmur [34], added to the 40 mm × 70 mm gel slab wells, and electrophoresed for 2.5 hours at 14 mA at 4°C.

Total Culturable Counts. Culturable counts were determined in triplicate by the standard plate count (SPC) procedure using the spread plate technique. Dilution blanks consisted of 15‰ marine salts solution. Plating medium was SWC (sea water complete) agar containing peptone (Difco), 5 g/liter; yeast extract (Difco), 0.5 g/liter [3]; and Instant Ocean to give a salinity of 15‰, pH 7.4.

Statistical Analysis of Data. Linear correlation analysis and multiple linear regression analysis were performed on the distribution data using SAS (Statistical Analysis System, Cary, North Carolina). Also, single factor analysis of variance (ANOVA) was conducted on distribution data by taxonomic groups using an HP-41CV computing device (Hewlett-Packard, Corvallis, Oregon) equipped with a standard 4k-byte statistics application pac (Hewlett-Packard). Program "xeq AVONE" was used.

Laboratory Induction Studies

Long-term Starvation in Closed Systems. Strains of vibrios (*V. cholerae* strains: CA 401, E 507, WF 113, WF 110, and WF 109; *V. fluvialis*: OSU 79, *V. damsela*: OSU 45, *V. parahaemolyticus*: ATCC 17082), and *Aeromonas sobria*: OSU 483 were grown in SWC broth overnight at 35°C. Cells were harvested by centrifugation, washed three times in carbon-free basal salts [3], and inoculated into 1 liter flasks containing basal salts solution at concentrations of 10^5 cells/ml (10^6 cells/ml for *V. cholerae*, E 507). The flasks were incubated at ambient temperature (20 ± 2°C) for up to 3 years. Samples were collected from flasks, and culturable counts were determined using the SPC procedure on SWC agar. SWC in the induction experiments was made with basal salts instead of Instant Ocean. Total direct counts were determined using the acridine orange direct counting procedure [20] with prestained 0.1 μm pore size Nuclepore filters, and MPNs were determined using the three-tube dilution series in SWC broth. The number of filterable bacteria was determined by passing 10 ml of the sample through sterile 25 mm Swin-loks, one fitted with a 0.2 μm, and another with a 0.4 μm pore size filter, diluting the filtrate in basal salts solution and performing the SPC and MPN procedures using SWC and SWC_o media. SWC_o was sea water complete agar or broth diluted with basal salts solution to give a final nutrient concentration of 5.5 mg/liter as peptone (5 mg) and yeast extract (0.5 mg). The broth and agar were designated the

Table 1. Number of ultramicrobacteria (0.2 μm filterable bacteria), total culturable counts (colony forming units—CFU), and selected water quality parameters of sites in Perdido Bay

Site	Salinity ‰	Degree of human impact on site	Number of ultramicro-bacteria/ml	CFU/ml	BOD mg/liter	Organic-N mg/liter	Total-P mg/liter
B	19.5[a]	Negligible	6	5×10^3	0.5	0.03	0.040
A	31.5	Light	2×10^{-1}	4×10^3	0.5	0.03	0.040
E	28.5	Moderate	5×10^{-1}	4×10^3	1.35	—	0.020
D	12	Great	1×10^{-1}	1.6×10^5	1.60	0.539	0.034
C	6	Greatest	0	1.6×10^5	1.30	0.478	0.053

[a] Data are expressed as mean values for entire study period
— = insufficient samples for mean to be representative

oligotrophic media. SWC_O agar was made with Noble agar (Difco). All regular strength media were incubated at 35°C for 4 days, and growth was observed as turbidity in the MPN tubes. In the oligotrophic medium (SWC_O broth), turbidity or cellular material (especially in the bottom of the tubes) was observed after 21 days. Also, tubes not displaying turbidity were checked. Each tube was streaked onto SWC and SWC_O agar and inoculated into SWC and SWC_O broth. Growth in/on any medium was considered a positive tube. Growth in SWC_O broth was determined by AODC (using 0.1 μm filters) immediately after inoculation and AODC after incubation for 21 days. Growth only on SWC_O broth and/or agar was considered to indicate an obligate oligotroph, and growth on both SWC and SWC_O agar and/or broth was considered a facultative oligotroph. All SWC_O agar plates were incubated in a moisture chamber to prevent drying. Figure 2 illustrates the procedure. (Replica plating from SWC to SWC_O and vice versa was omitted in this experiment.)

Continuous-Flow Starvation. One strain, *V. fluvialis,* was grown in SWC for 18 hours at ambient temperature, washed three times in basal salts, and inoculated into a 200 ml continuous-flow chamber containing basal salts solution. The inoculum size was 10^6 cells/ml and the flow rate was adjusted to 200 ml/24 hours (with the chamber volume kept at 200 ml). At selected intervals from 0 to 60 days, samples were taken for SPCs, AODCs, and MPNs as previously described. Samples were also filtered through 0.2 and 0.4 μm pore size Nuclepore filters and counted using the SPC, MPN, and AODC procedures as described and illustrated in Figure 2. Replica plating was also performed from the surface of the SWC agar onto SWC_O agar, and SWC_O agar onto SWC agar to determine the number of culturable obligate oligotrophs, facultative oligotrophs, and eutrophs. Those colonies capable of growing only on SWC_O agar but not SWC agar were reinoculated into SWC_O and SWC broth as an additional check for obligate oligotrophism.

Progressively Low-Nutrient Conditioning in Closed Systems. Strains of *V. cholerae* (WF 109), *V. fluvialis,* and *V. damsela* were first cultured in SWC broth for 2 days at ambient temperature. SWC broth in this experiment was made with deionized, distilled water and basal salts. One milliliter of this broth was transferred to a second SWC broth (medium 2) containing one-half the nutrient concentration (2.5 g peptone and 0.25 g yeast extract/liter) of the first medium. After incubation at ambient temperature for 21 days, 1 ml from medium 2 was transferred to medium 3 which contained one-half the nutrient concentration (1.25 g peptone and 0.125 g yeast extract/liter) of medium 2. Each organism was incubated into progressively more dilute medium until medium 12 (2.4 mg peptone and 0.24 mg yeast extract/liter) was reached. Medium 12, representing the lowest nutrient level, was considered the final medium. SPC and AODCs were determined before and after incubation to determine if growth occurred. Samples from medium 12, after incubation for 21 days, were filtered through 0.2 and 0.4 μm pore size filters, and bacteria in the filtrates counted using the SPC and MPN procedures as described and illustrated in Figure 2. Replica plating from SWC onto SWC_O and vice versa was also performed to determine the percent of culturable obligate oligotrophs and facultative oligotrophs.

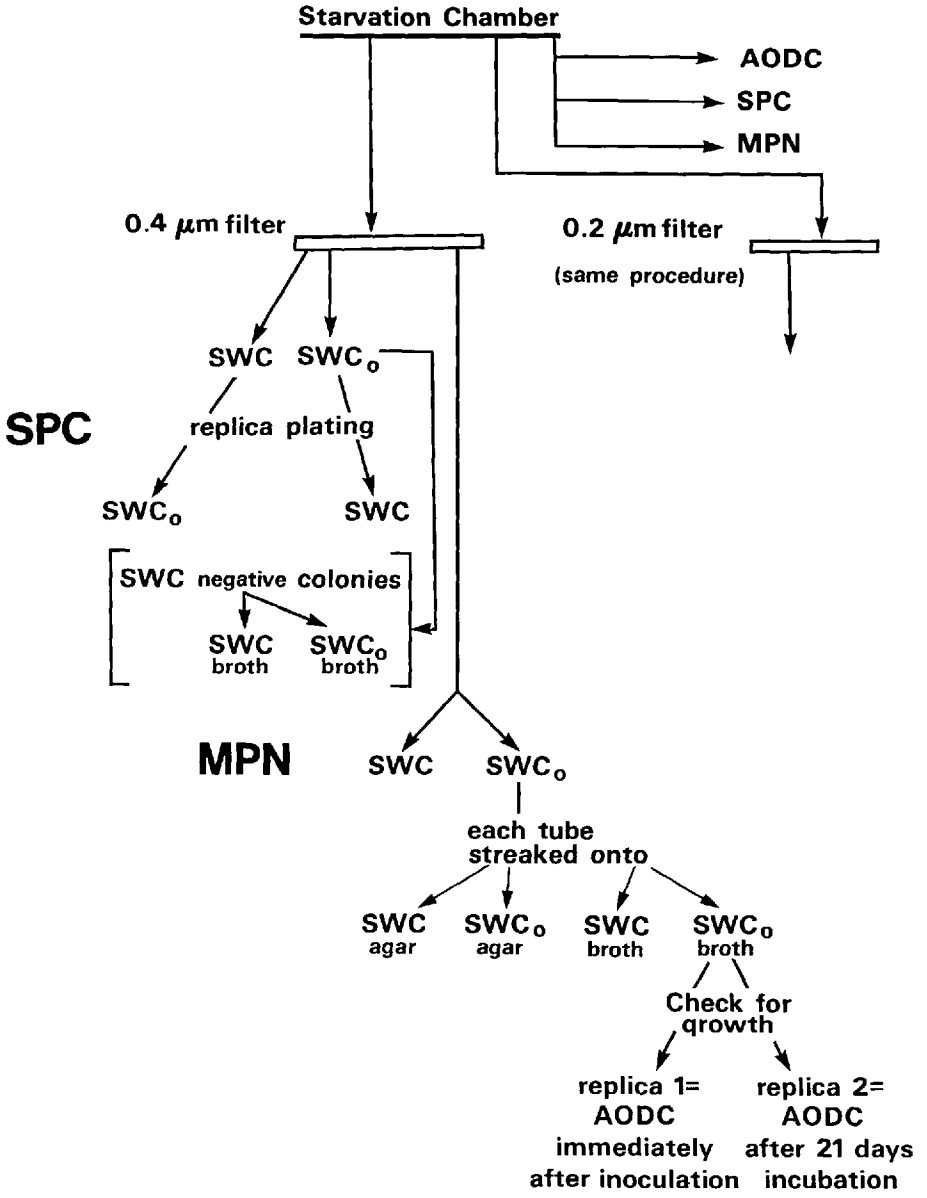

Fig. 2. Procedure for the determination of filterable cells; obligate oligotroph = growth only on/in SWC_O; facultative oligotroph = growth on/in SWC_O and SWC; eutroph = growth on/in only SWC.

Results

Distribution of Ultramicrobacteria

The total number of ultramicrobacteria correlated with the degree of human impact of the sites, water quality, and culturable counts (Table 1). In sites where

water quality was poor, where human impact was great, and where culturable counts were high (sites C and D), ultramicrobacteria were absent or scarce. However, in the relatively undisturbed area where water quality was high and culturable counts low (site B), ultramicrobacteria were present in the highest concentrations. Linear correlation analysis revealed that the inverse relationship among the number of ultramicrobacteria and culturable counts, BOD, organic-N, and total-phosphorus were significant at the 90, 95, 90, and 90% level of confidence, respectively. There were no significant correlations among total number of ultramicrobacteria and pH, salinity, temperature, total Kjeldahl-nitrogen, ammonium-nitrogen, and nitrate/nitrite-N levels. There were too few organic-carbon samples analyzed for the statistical analysis to be valid. Multiple regression analysis revealed no further significant interrelationships.

During the 1-year study period, 121 ultramicrobacterial strains from the four sites were isolated and 85 strains were characterized (Table 2). The total population of ultramicrobacteria was divided among three major groups: *Vibrio* and *Listonella* sp. (microvibrios), *Pseudomonas* sp. (micropseudomonads), and a heterogenous group consisting predominantly of *Acinetobacter, Flavobacterium, Alcaligenes,* and *Allomonas.* The majority of the isolates were oxidase positive (94%), catalase positive (88%), lactose negative (92%), string test positive (84%), motile (95%), and, with the exception of a single isolate, all were gram-negative.

Although it has been suggested that some ultramicrobacteria may represent new classes of bacteria [40], all the ultramicrobacteria isolated in this study were identified as belonging to previously established genera. Based on phenotypic characterization and GC mol%, the isolates were identified, and the dominant species were of the genera *Vibrio* and *Listonella* [30] (Table 2). Of the species represented by these ultramicrobacterial populations, the majority were *V. anguillarum, V. hollisae,* and *V. fluvialis.*

Speciation of members of the genus *Pseudomonas* was not attempted, but it was apparent from their phenotypic profiles that relatively few species of *Pseudomonas* were present. Those fitting the phenotypic profile of *P. putrifaciens,* however, were cited, although it should be pointed out that this species is considered *insertae sedis* due to a lack of correlation with established properties of the genus *Pseudomonas,* not the least significant of which was an inappropriate GC mol% (47.7 mol%). In the most recent Bergey's manual [29], the organism's taxonomy still remains uncertain, but it has been reassigned to the genus *Alteromonas.*

Correlations between the occurrence of ultramicrobacteria of selected families and (1) salinity, (2) seasonality, and (3) extent of human impact on site waters (Table 3) revealed certain interesting relationships. It was evident from analysis of variance of the distribution of the three groups of ultramicrobacteria at various salinity ranges that, at a 90% confidence level, the distribution of microvibrios, but not of micropseudomonads, varied significantly with salinity, $F = 3.00$ (Table 3A). Statistical analysis indicated that microvibrios occurred significantly more frequently at salinities less than 14‰. The occurrence of each of the major groups within selected salinity ranges was also analyzed (Table 3B). Although there were differences in the mean percentages of members of the major groups, these differences were not significant at the 90% confidence level, $F = 1.44$ and 1.85.

Table 2. Taxonomic profile of the ultramicrobacteria isolated from Perdido Bay

Taxa	GC mol %	Number of strains isolated
Alcaligenes sp.	59.6	3
Allomonas sp.	42.5	3
Flavobacterium sp.	60.0	3
Acinetobacter sp.	—	2
A. calcoaceticus	38.7	2
Aeromonas sp.	—	2
Alteromonas sp.	51.2	1
Cytophaga sp.	42.3	1
Herpetosiphon sp.	52.7	1
Photobacterium sp.	—	1
Staphylococcus sp.	—	1
Pseudomonas sp.	60.1	12
P. angustum	—	2
P. putrifaciens	47.7	8
Vibrio sp.	38.6	6
V. anguillarum[a]	44.9	5
V. alginolyticus	—	2
V. campbellii	—	1
V. damsela[a]	—	3
V. fluvialis	—	4
V. harveyi	—	3
V. hollisae	49.5–48.0	12
V. ordalii	—	1
V. parahaemolyticus	—	2
V. pelagius	—	1
V. vulnificus	—	2

— = not performed
[a] *Listonella* sp. [30]

When the effect of seasonality on family distribution was statistically evaluated, certain significant interactions among season and certain taxa were observed. Comparison of the abundance of microvibrios and micropseudomonads within season (winter; summer) demonstrated significant seasonal effects at the 95% level of confidence, F = 4.02 and 4.08 (Table 3B). Microvibrios dominated the population in both winter and summer months, while micropseudomonads appeared to be inhibited during the winter. The net effect, an ultramicrobacterial population dominated by microvibrios in the winter, was in contrast to the seasonal distribution of rapidly metabolizing vibrios, which have been shown to reach peak populations in the water column in the warmer summer months [9, 21].

The relative distribution of microvibrios and micropseudomonads between sites was not found to be significant (Table 3B). However, analysis of the distribution of the major groups of ultramicrobacteria within each site demonstrated that, at site E (moderate impact), significant effects on distribution

Table 3. Effect of salinity, seasonality, and degree of human impact on the distribution of the three groups of ultramicrobacteria

A. Statistical analysis by bacterial family

	Salinity ‰				Season			Degree of impact			
	>25	15–24	<14	F	Summer	Winter	F	Negligible (site B)	Light (site A)	Moderate (site E)	F
Pseudomonadaceae	35[a]	34	0	0.73[b]	41	18	1.15[b]	45	47	8	1.15[b]
Vibrionaceae	50	44	100	3.00[c]	54	55	0.04[b]	45	32	68	1.32[b]

B. Statistical analysis by environmental parameter

Salinity ‰	Pseudomonadaceae	Vibrionaceae	Other families	F
>25	35	50	15	1.44[b]
15–24	34	44	22	1.85[b]
Season				
Summer	41	54	5	4.02[c]
Winter	18	55	27	4.08[c]
Degree of impact				
Negligible	45	45	10	1.80[b]
Light	47	32	21	1.99[b]
Moderate	8	68	24	4.00[c]

[a] Percent of population
[b] Where F is less than critical, distribution is random
[c] Where F is greater than critical, significant interaction at the 90% level of confidence or above

were attributable to water quality, F = 4.00 (Table 3B). Microvibrios predominated at site E, whereas micropseudomonads were sparse at this site.

Induction of Ultramicrobacteria

To determine if ultramicrobacteria could be induced by nutrient deprivation, laboratory studies were performed wherein strains of vibrios were exposed to low nutrients. Three approaches were used: (1) seven vibrio strains (plus an *Aeromonas* sp.) were exposed to sudden low nutrients in closed flasks; (2) one *Vibrio* species was exposed to low nutrients in a continuous-flow system; and (3) three vibrio strains were exposed to progressively lower nutrients by passage from high nutrient medium to low nutrient medium.

Table 4 presents the results of the laboratory studies in which the strains were exposed to long-term nutrient deprivation in closed flasks. All strains examined were able to survive for long periods of time, and 0.2 μm filterable cells were recovered. Four vibrio strains exhibited filterable cells that were obligate oligotrophs, while all vibrio strains exhibited filterable cells that were facultative oligotrophs. Of interest was the observation that the MPN technique,

Table 4. Recovery of *Vibrio* and *Aeromonas* species from basal salts solution after long-term nutrient starvation in closed systems

Organism[a]	Culturable counts[b]		Direct counts[b]	Number of 0.2 μm filterable cells[b]					
				Culturable counts		Most probable number		Facultative oligotrophs	Obligate oligotrophs
	SPC	MPN	AODC	SWC[c]	SWC_o[d]	SWC[c]	SWC_o[d]		
V. cholerae CA401	2.4×10^3	9.0×10^3	2.0×10^5	1.7	<1	0.5	1.2×10^2	1.8	0
V. cholerae E507	5.6×10^4	1.9×10^5	1.5×10^6	3.5×10^4	1.0×10^3	4.6×10^4	6.6×10^4	1.7×10^3	0
V. cholerae WF 113	1.4×10^5	2.9×10^5	5.5×10^5	<1	<1	<1	1.0×10^3	1.8×10^1	0
V. cholerae WF 109	2.0×10^5	4.1×10^5	8.8×10^5	<1	<1	<1	2.1×10^3	4.0×10^1	2.0
V. fluvialis	5.5×10^4	3.2×10^5	1.1×10^6	4.0×10^2	3.4×10^2	4.6×10^2	1.5×10^4	3.6×10^2	1.0×10^2
V. parahaemolyticus	4.5×10^4	3.8×10^5	1.0×10^6	3.1×10^2	3.0×10^2	3.9×10^2	1.1×10^4	3.2×10^2	1.7×10^2
V. damsele[e]	9.2×10^4	3.1×10^5	9.1×10^5	2.0×10^2	2.2×10^2	2.8×10^2	1.3×10^4	2.7×10^2	1.1×10^2
A. sobria	1.7×10^3	2.9×10^3	1.1×10^4	<1	<1	<1	4.6×10^1	0	0

[a] Strain CA401 was starved for 3 yrs; E507 and *V. parahaemolyticus* 1 yr; all others, 2.5 yrs
[b] Data are expressed as number of cells/ml
[c] SWC = sea water complete media
[d] SWC_o = sea water complete oligotrophic media
[e] *L. damsela* [30]

Table 5. Recovery of *Vibrio fluvialis* in low-nutrient concentrations in a continuous-flow system after 30 days

Cell fraction	Direct counts	Culturable counts[a]		Most probable number	
		SWC	SWC_o	SWC	SWC_o
Total cells	1.9×10^5	1.5×10^5	1.4×10^5	3.7×10^6	1.5×10^5
0.4 μm filterable cells	4.8×10^4	3.1×10^3	2.0×10^3 (90%)[b] [10%][c]	2.4×10^3	2.4×10^3 (85%)[b] [15%][c]
0.2 μm filterable cells	0	0	0	0	0

[a] Data are expressed as number of cells/ml
[b] Percent of population that also grew on SWC (facultative oligotrophs)
[c] Percent of population that grew only on SWC_o (obligate oligotrophs)

using SWC broth, generally recovered higher numbers of cells than the SPC method for unfilterable cells and higher numbers than the culturable counts for 0.2 μm filterable cells. One possible explanation is that some of the cells may be unable to withstand the initial stress of agar plating and/or the osmotic stress of high nutrients. Likewise, MPNs were also much higher than SPCs using SWC_O. This suggests that some filterable cells are unable to grow on SWC_O agar. While SWC_O agar contains only 5.5 mg/liter utilizable nutrients, it also contains 2.0% Noble agar which may inhibit the growth of (some) obligate oligotrophs.

In the second set of experiments, starvation consisted of the exposure of *V. fluvialis* to nutrient deprivation using a continuous-flow system. The organism was able to maintain a relatively large population at low nutrient levels over time, and after 30 days, 0.4 μm filterable (but not 0.2 μm filterable) oligotrophic cells were present (Table 5). Replica plating revealed that all the colonies (100%) of the 0.4 μm filterable population present on SWC could grow on SWC_O but that only 90% of the colonies that developed on SWC_O could grow on SWC, indicating that 10% of the cells in the population were obligate oligotrophs. Upon reinoculation into SWC_O and SWC broth, these colonies were confirmed to be obligate oligotrophs. From MPNs using SWC_O, 100% of the population was capable of regrowth in SWC_O broth, but only 85% was capable of growth in SWC, suggesting that obligate oligotrophs comprised 15% of the population. After 60 days, only a few 0.2 μm filterable cells/ml were recovered and only from SWC_O broth (data not shown). They were facultative oligotrophs.

In the third set of experiments wherein starvation was accomplished by progressive exposure to low nutrients in closed flasks, the three vibrio strains examined were capable of growth at all nutrients levels (data not shown) and capable of producing 0.4 μm filterable cells (Table 6). Although less than 10 cells/ml of 0.2 μm filterable cells were recovered from medium 12 for all strains, approximately 1% of the culturable population (using SPCs) consisted of 0.4 μm filterable cells for *V. cholerae* and *V. fluvialis*, whereas only 0.01% of the population was 0.4 μm filterable for *V. damsela*. A large portion of the 0.4 μm filterable, culturable population (using both SPCs and MPNs) consisted of facultative oligotrophs (approximately 90%), while a range of 10–20% of the population was obligately oligotrophic.

Table 6. Percent of total population that was 0.4 μm filterable and oligotrophic when vibrios were exposed to progressively lower nutrient conditioning

Organism	Number of 0.4 μm filterable cells[a] (%)	Number of oligotrophs[b] (%)	
		Facultative	Obligate
V. cholerae (WF 109)	1.0	90 (86)	9.9 (14)
V. fluvialis	1.5	84 (82)	15.9 (18)
V. damsela[c]	0.01	87.4 (88)	12.5 (12)

[a] Percent of total culturable counts calculated using SPCs in medium #12 that was 0.4 μm filterable
[b] Percent of total 0.4 μm filterable culturable counts calculated using SPCs (and MPNs) in oligotrophic (#12) medium
[c] *L. damsela* [30]

Discussion

The results of the distribution and characterization study showed that (1) the presence of ultramicrobacteria in the estuary strongly correlated to low nutrient levels in the water column; (2) the presence of certain groups of ultramicrobacteria was related to salinity and temperature; and (3) the ultramicrobacteria were marine or estuarine strains of previously identified taxa.

The results of the laboratory studies showed that the aquatic vibrio strains examined were (1) capable of long-term survival in nutrient depleted waters; (2) capable of forming 0.2 or 0.4 μm filterable cells; and (3) capable of forming filterable cells that were oligotrophic. Gradual and short-term nutrient stress resulted primarily in 0.4 μm filterable cells which were facultative oligotrophs, whereas long-term nutrient stress in closed systems had the most dramatic effect resulting in many 0.2 μm filterable cells, some of which were obligate oligotrophs. Since the laboratory systems used were sterile and involved only single species, it might be argued that such systems do not really resemble natural ones. However, the results of the laboratory studies in combination with the field study lend support to the idea that certain aquatic bacteria, especially the vibrios, appear to be capable of forming very small cells when nutrient levels are low and that some of these cells are capable of growth at an extremely wide range of nutrient levels. It would certainly seem logical to assume that such properties would be advantageous to an organism in an environment characterized by low nutrients and nutrient fluctuation.

The concept of two broad classes of aquatic bacteria, i.e., oligotrophs and eutrophs, is being challenged [35, 43]. If one modifies the model of only two physiological stages, currently used to describe aquatic bacteria, to include many physiological stages along a continuum, such a model might be more consistent in terms of describing the phenomenon of ultramicrobacteria as forms resulting from nutrient deprivation. Since it is well recognized that the limits of a cell's size (largeness or smallness) are genetically controlled, but that within those limits, nutrient levels can determine bacterial size, it is entirely possible that ultramicrobacteria (at least some portion of them) may represent forms that

are in some intermediate position on a continuum with "large, healthy, metabolically active" cells on one end, "small, metabolically reduced" cells along the continuum, and "dead" cells on the other end.

In a previous study, some of 0.2 μm filterable bacteria in the vibrio group were obligate oligotrophs but could be "trained" to grow on high nutrient medium [31]. This observation coupled with the fact that "healthy" indigenous aquatic strains of vibrios could be induced to form filterable obligate oligotrophs and that ultramicrobacteria in the environment appear to be present when nutrient levels are low, suggests that this continuum model might be a valid one. Furthermore, some of the obligate oligotrophs induced in these laboratory studies could also be "retrained" to grow on high nutrient medium using the previously described methods [31]. The fact that aquatic organisms have great flexibility and adaptiveness with respect to their nutrient uptake abilities has been discussed in detail [17]. This review boldly states that "an organism . . . is very much a product of its environment." The observation that within a population cells could go from an [3] eutrophic nutritional state to a facultative oligotrophic one, to an obligate oligotrophic state with a concomitant reduction in size upon nutrient deprivation (and back again with nutrient addition), is not inconsistent with the concept of a continuum model. The results of this study do not attempt to eliminate totally the possibility that obligate oligotrophs exist in the aquatic environment autochthonously and that such organisms may never adapt to high nutrients or become larger cells. However, the results of this study do suggest that some portion of the obligate oligotrophic ultramicrobacteria results from eutrophic organisms (especially vibrios) under the stress of nutrient deprivation.

It has been well argued that reductive division and dwarfing are survival strategies useful to aquatic organisms [23, 28, 37, 41], but whether such strategies are common among all aquatic bacteria or are limited to vibrios and a few other aquatic genera remains to be demonstrated. However, the fact that many vibrios can form microvibrios, that these forms can apparently survive for long periods of time on extremely low nutrients, and that they can be revived when nutrient conditions are more favorable, carries important and significant public health implications in terms of the epidemiology of the potential human pathogenic vibrios.

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