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Isolation and Characterization of Ultramicrobacteria from a Gulf Coast Estuary

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Viable bacteria were recovered from estuarine waters passed through a 0.2-μm polycarbonate membrane filter. The recovery method included the use of a dilute nutrient broth for primary enrichment followed by conditioning of the organism to a dilute nutrient solid medium. These bacteria were gram-negative rods and coccobacilli having an NaCl requirement and, upon initial culturing, low nutritional requirements. In response to increased nutrient preparations, these microorganisms underwent an increase in size and growth rate, giving rise to visible colonies. Phenotypic characterization suggests that species of *Vibrio*, *Aeromonas*, *Pseudomonas*, and *Alcaligenes* were among the isolates. The abundance and the nutritional requirements of these ultramicrobacteria imply that they represent a class of microorganisms which have successfully adjusted to poor nutrient conditions.

Ultramicrobacteria were first defined by Torrella and Morita (23) as bacteria less than 0.3 μm in diameter which had a very slow growth rate when inoculated onto a nutrient-rich agar surface and which did not increase significantly in cell size on this medium. Since filterable bacteria (1) are generally regarded as those capable of traversing a 0.45-μm membrane filter, it is logical to assume that the filterable bacteria would include the class defined as the ultramicrobacteria.

The isolation of membrane-filterable bacteria has proceeded rather sporadically since the first report of their existence by Oppenheimer in 1952 (14). Apparently, much of the difficulty encountered in the isolation of these bacteria has been caused by use of media inappropriate to their growth (4, 5). A significant advance was made toward the solution of this problem by Torrella and Morita (23), who employed a less nutrient-rich solid medium containing a low concentration of peptone and yeast extract (0.175%). Although they were able to obtain growth from some of their isolates, others either failed to respond or grew for several cell divisions and ceased further reproduction.

Tabor et al. (21) demonstrated that bacteria capable of passing through a 0.45-μm membrane filter were ubiquitous in the deep sea. These filterable bacteria were isolated by using standard media such as marine agar, and since full-strength nutrient medium was employed in the isolation, it is probable that they do not represent true ultramicrobacteria as defined by Torrella and Morita (23). As a result of their study, some important observations as to the role of size in the adaptation to starvation systems were presented. Tabor et al. (21) proposed that marine bacteria confronted with conditions of low solutes undergo certain changes. Among these are a decrease in volume and a change in morphology. They further suggested that these changes initiated at the onset of starvation are associated with the depletion of cellular reserves and that the nature of the marked decrease in volume is a function of the processes of scavenging nonessential cellular components and material.

Because of the recent interest in the problem of dormancy in marine microbial systems, considerable attention is now being focused on the filterable bacteria and ultramicrobacteria. Many investigators (7, 9–12, 20, 21, 23) have concluded that the little-studied filterable bacteria may represent the true autochthonous bacteria of the sea. Characterization of these microorganisms, however, had remained elusive until Tabor et al. (21) classified a group of 0.45-μm filterable bacteria taken from the Atlantic Ocean. In contrast to the filterable bacteria, isolation of the ultramicrobacteria has been sufficiently problematic that, to date, no published account of their characterization is available.

It was, therefore, the purpose of this study to examine the warmer waters of a Gulf Coast estuary for the presence of ultramicrobacteria, to attempt to isolate these organisms, and to phenotypically characterize them. Studies are also under way to examine other aspects of their ecology and physiology.

MATERIALS AND METHODS

Sample collection. Water samples were taken from approximately 10 cm below the surface at four sites located in Perdido Bay, Ala. (Fig. 1), during the months of April, May, and June 1981. Sites were selected for areas less impacted by human activities and representing a wide range of salinities. Samples were collected in sterile bottles and returned to the laboratory in insulated containers. In no case was the interval between collection and processing greater than 3 h.

Filtration. All water samples were routinely filtered through Whatman no. 1 filters (W & R Balston Ltd., London) to remove particles which would obstruct the Nuclepore filters. Samples were then passed through a 25-mm Swin-Lok (Nuclepore Corp., Pleasanton, Calif.) fitted with a 0.2-μm pore size Nuclepore polycarbonate filter by means of a sterile 30-ml disposable Plastipak syringe (Becton, Dickinson & Co., Rutherford, N.J.) with very gentle pressure on the syringe piston. The use of gentle pressure was essential to avoid the risk of rupturing or damaging the membrane filter. The Swin-Lok and filter were routinely assembled and autoclaved intact for 10 min at 15 lb (ca. 6.8 kg) of pressure. Samples (5 ml) of the resulting filtrate were collected dropwise in sterile test tubes containing 1.0 ml of Lib-X broth (2). Test tubes (20 mm in diameter) were employed because they provided for a tight fit with the Swin-Lok base, reducing the likelihood of contamination. The resulting diluted inocula were incubated at 21 and 35°C and observed for growth at 24-h intervals. Tubes were not discarded until no growth was evidenced for a period of 21 days. After each filtration the Swin-Lok was carefully disassembled, and the Nuclepore filter was inspected for proper seating on the O-ring and for evidence of mechanical failure. Of particular concern was the mechanical integrity of the Swin-Lok apparatus, seating of the polycarbonate filter on the filter stage, effective gasket seal, and ability of the filter to endure autoclave temperatures without becoming distorted.

Water samples were also examined by the epifluorescent techniques of Hobbie et al. (8). A 0.2-μm Nuclepore filter was used to collect the bacteria. A Zeiss standard 18 microscope was used with a 450- to 490-nm band pass filter, an FT 510 beam splitter, an LP 520 barrier filter, and a 100-W halogen lamp.

Controls were established by duplicating the entire filtration procedure each time samples were taken by using 5-ml suspensions of *Hafnia* spp. (an environmental isolate). In addition, 75 ml of a suspension containing 10^8 cells of *Salmonella* spp. (environmental isolate) was filtered, by the same procedure, into 25 ml of a 4X strength Lib-X broth.

Isolation of ultramicrobacteria. Inocula showing growth were streaked onto a low-nutrient solid medium, brain heart infusion-estuarine salts agar (BH-ES) having the following composition: 3.7 g (1/10 strength) of brain heart infusion (Difco Laboratories, Detroit, Mich.), 20 g of agar (Difco), 15 g of Instant Ocean marine salts (Aquarium Systems, Mentor, Ohio), and deionized water to make 1 liter (pH 7.5). The plates were incubated at 21 and 35°C for 48 h, and cultures were transferred to fresh media and again incubated. It was generally necessary to repeat this process four or five times, after which colony development was sufficient to allow selection of single discrete colonies for characterization.

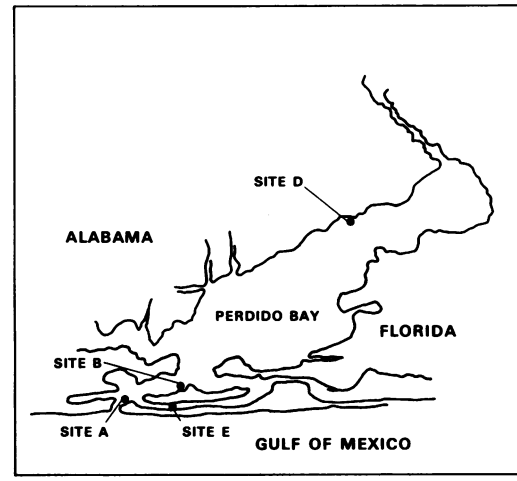

FIG. 1. Sampling sites of estuarine waters in Perdido Bay.

Characterization of ultramicrobacteria. Carbohydrate utilization was determined by using phenol red broth base (Difco) and the appropriate sugar. Decarboxylase reactions were tested by using decarboxylase base medium (Difco). All media employed, including MR-VP medium (Difco) and OF medium (Difco), were diluted eightfold with an aqueous solution of 15‰ Instant Ocean marine salts. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) disks (BBL Microbiology Systems, Cockeysville, Md.) and oxidase disks (Difco) were used for the *o*-nitrophenyl-β-D-galactopyranoside and cytochrome oxidase reactions. NaCl requirements were determined by testing for growth in an NaCl-free solution consisting of 1.5 g of Trypticase (BBL) and 1.5 g of yeast extract (BBL) with sufficient deionized water to make 1 liter. Susceptibility to the vibriostat O/129 (2,4-amino-6,7-diisopropyl pteridine) (Sigma Chemical Co., St. Louis, Mo.) was determined by using concentrations of 50 and 150 μg/ml in BH-ES broth. Motility was determined by the hanging drop method with the aid of a Wild (Heerbrugg, Switzerland) phase-contrast microscope. H_2S production was determined by the presence of a black precipitate of the $FeSO_4$-containing BH-ES agar over a period of 96 h (14, 15). Tryptophan deaminase determinations were by the method of Singer and Volcani (17), with the modification that the organisms were inoculated into the tryptophan broth and incubated overnight at 20°C. Three percent hydrogen peroxide was employed in the catalase test (22). Several identification schemes (6, 16, 19) were used to determine genera.

Preparation of growth curves. The isolates were grown on BH-ES at 35°C for 4 days. Colonies were removed, and a 0.9% NaCl suspension was made. Samples (0.1 ml) of the suspension were inoculated into a series of various concentrations of Trypticase in natural estuarine water (15‰). Incubation was carried out at 35°C for 36 h. Turbidity was measured by the use of a Klett-Summerson colorimeter (Klett Manufacturing Co., New York) fitted with a Klett no. 66 red filter.

RESULTS

Visual confirmation that the bacteria were present in the waters was made by observing them under fluorescent microscopy. Numerous cocci and coccobacilli approximately 0.2 μm in size were caught on the filter and could be observed.

No growth was obtained in any of the control inocula, suggesting that the filter apparatus was retaining cultured cells. Viable ultramicrobacteria were recovered from all water samples taken from the Perdido Bay sites, after filtration through 0.2-μm pore diameter polycarbonate filters.

Of the 27 isolates, 89% were initially incapable of growth in any of several full-strength nutrient-rich broths, including Trypticase soy broth plus marine salts, brain heart infusion broth plus marine salts, marine 2216 broth, thioglycolate broth plus marine salts, or any full-strength carbohydrate test medium. Furthermore, no isolate was capable of a single positive reaction in full-strength biochemical media or on API-20E test strips (Analytab Products, Plainview, N.Y.). This was found to be true even when the media were prepared (or the API-20E strips were inoculated) with appropriately elevated NaCl concentrations. To determine whether a lower concentration of nutrients would provide the proper conditions for growth, several dilutions of various media were prepared. The optimum concentration was observed to be an eightfold dilution of full strength (Table 1). This dilution permitted the organism to grow while providing enough indicator to allow classical color reactions to be observed in the test media.

TABLE 1. Response of five Perdido Bay ultramicrobacteria (UM 6, UM 7, UM 8, UM 9, UM 106) to various dilutions of selected media

Medium[a]	Growth[b] at the following dilution of medium:			
	Full strength	1/2	1/4	1/8
PRBB + Dex	0	0	G	A
PRBB + Lac	0	0	G	–
PRBB + Suc	0	0	G	A
PRBB + Man	0	0	G	A
Decarboxylase base	0	0	G	R
TSB	0	0	G	G
MR-VP	0	0	G	R
OF medium + Dex	0	0	0	r

[a] PRBB, Phenol red broth base; Dex, dextrose; Lac, lactose; Suc, sucrose; Man, mannose; TSB, Trypticase soy broth; MR-VP, methyl red Voges-Proskauer medium.

[b] 0, No growth; G, growth; A, acid production; –, no reaction; R, positive reaction; r, weak positive reaction.

The fact that the microorganisms isolated were capable of growth in dilute broths, but not in full-strength broths, suggests that the organisms were being inhibited by rich nutrient concentrations. Two of the isolates, UM106 and UM403, were examined for their ability to grow at a range of concentrations of Trypticase in 15‰ marine salt water. The growth responses after 36 h at the various concentrations are presented in Fig. 2. A range of concentrations of both yeast extract and proteose peptone produced similar curves. In each case, the optimum concentration for growth of the freshly isolated ultramicrobacteria was 0.04 to 0.05%.

Since characterization requires the isolation of a single-cell clone colony of the organism, and the simplest procedure to obtain such a culture is by the use of streak plates, the problem of obtaining growth on a solid medium was confronted. In general, agars were found to be somewhat more inhibitory than broths. Even when using low-nutrient agar media such as BH-ES, it was necessary to condition the organism to the medium over a period of several days. This was achieved by heavy inoculation of an area of the BH-ES agar approximately 1 cm in diameter with the enrichment broth, followed by the transfer of the isolate at 48-h intervals to new media until growth was sufficiently heavy to allow proper streaking for isolation.

Isolate UM106, after undergoing nutrient conditioning on BH-ES agar for a period of 6 weeks, was reexamined for its ability to grow at different nutrient levels. Growth responses to various concentrations of Trypticase were again determined and compared with those obtained shortly after its collection. The results of adaptation to

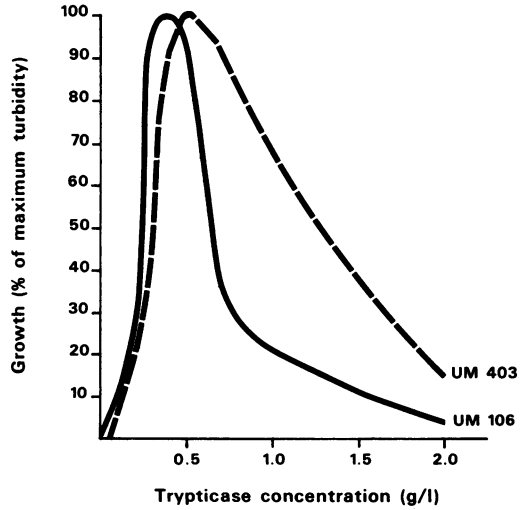

FIG. 2. Growth response of isolates UM403 and UM106 in Trypticase broth.

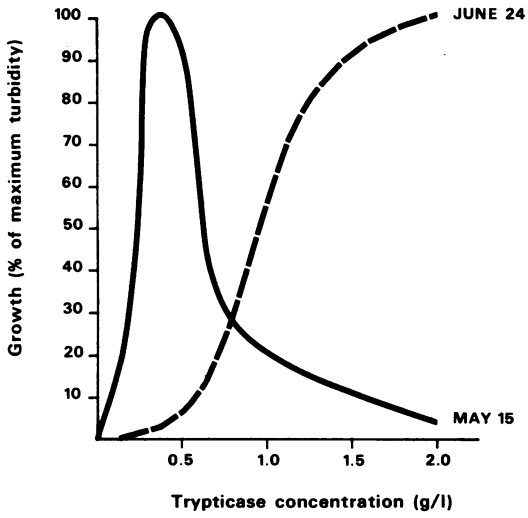

FIG. 3. Growth response of UM106 newly isolated (May 15) and after 40 days of adapting on culture media (June 24).

nutrient-rich media are reflected in the pronounced shift in the isolate's response to low concentrations of nutrient (Fig. 3).

Adaptation to nutrient-rich media was accompanied by a pronounced increase in size. Phase-contrast microscopic examination of those broth inocula giving rise to visible turbidity within 48 h indicated microorganisms of a size range of about 0.3 to 0.4 by 1.0 to 1.5 μm. Those microorganisms typical of broth inocula showing turbidity only after incubation for a week or more generally appeared as extremely slender rods, the widths of which were at the limit of the resolving power of the microscope (0.17 μm), with a length of 0.5 to 0.8 μm.

Regardless of the initial response to broth media, nutrient conditioning sufficient for the development of discrete 1- to 2-mm colonies on reduced-nutrient solid medium (BH-ES) was accompanied by the development of microorganisms of a "normal" size, 0.5 by 2.0 to 2.5 μm.

As can be seen from the profile of biochemical and phenotypic properties (Table 2), the majority of the isolates were oxidase positive and catalase positive. All were gram negative, and with a single exception, all were motile. With three exceptions, every isolate was string test positive. It has been reported that only certain *Vibrio* spp. produce positive string test reactions (18). However, results from our laboratory indicate that some phenotypic environmental *Aeromonas* spp. are also positive. Because of the high incidence of positive reactions produced by the Perdido Bay isolates, the string test may be a meaningful characteristic, although the significance of the test is unclear at this time.

DISCUSSION

Because of their size and the environment from which they were collected, the possibility that the Perdido Bay isolates might include host-independent strains of *Bdellovibrio* (3) was suggested. *Bdellovibrio* are microorganisms about which remarkably little has been documented. They have not been demonstrated to utilize carbohydrates, and many of their phenotypic characteristics remain unidentified. It is generally recognized, however, that *Bdellovibrio* are either catalase positive and O/129 sensitive or catalase negative and O/129 resistant. Furthermore, the host-independent strains are characterized as nonfermentative, nonmotile, catalase-negative organisms existing as curved rods, coils, and long filaments with an average length of 10 μm (3). As this offered little correlation with the Perdido Bay isolates, and since the addition of the isolates to cultures of *Pseudomonas, Vibrio*, and species of *Enterobacteriaceae* yielded no indication of parasitism, the possibility that the isolates represented strains of *Bdellovibrio* was discarded.

In their study, Torrella and Morita (23) defined ultramicrobacteria to include three criteria: (i) bacteria which had a diameter of 0.3 μm or smaller; (ii) bacteria (initially) incapable of growth on nutrient-rich solid media; and (iii) bacteria which did not increase in size upon ultra-microcolony formation. The information presented here indicates that the Perdido Bay isolates share the first two criteria. The third criterion, however, was not addressed in this investigation since the cells, enriched first in low-nutrient preparations and then slowly conditioned to low-nutrient solid media, eventually gave rise to visible colonies. Size, response to nutrient, and the environment from which they were collected suggest that the Perdido Bay isolates are ultramicrobacteria.

It has been suggested by a number of investigators (11, 12, 21, 23) that adaptation to very low solute conditions (starvation) involves a series of processes including depletion of cellular reserves and degradation of nonessential cellular material which is linked to morphological changes in the cell such as rounding up and reduction in volume. Much of the critical work on the effects of starvation conditions has been done by Novitsky and Morita (13). They demonstrated, using the marine isolate ANT-300, that after 7 days of starvation in seawater, endogenous respiration was reduced by more than 99%. It was argued that during evolutionary selection for the ability to maintain viability during nutrient stress, reduction in respiration may have emerged as the means by which macromolecular degradation is controlled. In another study (12)

TABLE 2. Biochemical and phenotypic profile of the Perdido Bay ultramicrobacteria

Site	Isolate	Reaction to the following tests[a]:																							Probable genus
						O/129												Acid from:							
		ONPG[b]	Catalase	Oxidase	Motility	50 μg/ml	150 μg/ml	LDC	ODC	TDA	Voges-Proskauer	NaCl requirement	20°C	35°C	Citrate	Dextrose fermentation	Dextrose	Mannose	Sucrose	Lactose	Pigment	String test	H_2S production	
A	UM6	+	+	−	+	+	+	+	+	−	−	+	+	+	−	−	+	−	−	−	−	+	−	*Vibrio*
A	UM7			+								+	+	+						−	−			(Lost)
A	UM8	−	+	+	+	+	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	+	+	*Vibrio*
A	UM9	+	−	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	−	+	−	*Vibrio*
A	UM10	−	+	+	+	+	+	−	+	−	−	+	+	+	−	+	+	+	+	−	−	+	−	*Vibrio*
A	UM11	+	w	−	+	+	−	+	+	−	−	+	+	+	−	−	w	−	−	−	+	+	−	*Vibrio*
A	UM12			+			+					+	+	+						−	−		−	(Lost)
A	UM13	−	+	+	+	+	−	+	+	−	0	+	+	+	+	−	−	−	−	−	−	−	−	*Alcaligenes*
A	UM14	−	+	+	+	+	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−	+	−	*Vibrio*
A	UM15	−	+	+	+	+	−	+	+	−	0	+	+	+	−	−	−	−	−	−	−	+	−	*Vibrio*
A	UM16	−	+	+	+	+	−	+	+	−	0	+	+	+	−	−	−	−	−	−	−	+	−	*Vibrio*
A	UM17	−	+	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	*Alcaligenes*
A	UM18	−	+	+	+	+	−	+	+	−	0	+	+	+	−	−	−	−	−	−	−	+	−	*Vibrio*
B	UM106	−	−	−	−	+	+	+	+	−	−	+	+	+	+	−	+	+	+	−	−	+	−	Unknown
B	UM108	−	+	+	+	+	−	−	−	−	+	+	+	+	−	+	+	−	+	−	−	+	+	*Vibrio*
B	UM109			+								+	+	+						−	−			(Lost)
B	UM110	−	+	+	+	+	+	−	+	−	−	+	+	+	−	−	+	+	−	−	−	−	−	*Pseudomonas*
B	UM111	−	w	+	+	+	+	−	+	−	−	+	+	+	+	+	+	+	−	−	−	+	−	*Vibrio*
B	UM112	−	+	+	+	+	+	−	+	−	−	+	+	+	+	+	+	+	−	−	−	+	−	*Vibrio*
B	UM113	−	+	+	+	+	+	−	−	−	−	+	+	+	−	+	+	+	−	−	−	+	−	*Vibrio*
B	UM114	−	w	+	−	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	−	+	−	*Pseudomonas*
D	UM301	−	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+	+	+	−	−	+	−	*Aeromonas*
D	UM302	−	+	+	+	+	+	−	+	−	−	+	+	+	−	+	+	+	+	−	−	+	−	*Vibrio*
D	UM303		+		+						−	+	+	+	+		+	+	+	−	−			(Lost)
D	UM304	−	w	+	+	+	+	−	+	−	−	+	+	+	−	+	+	−	−	−	−	+	−	*Vibrio*
E	UM405	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+	w	+	−	+	+	+	*Aeromonas*
E	UM406	+	w	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−	+	+	+	*Aeromonas*

[a] +, Positive reaction; −, negative reaction; w, weak reaction; 0, no growth; space, organism was lost before tests could be completed. LDC, Lysine decarboxylase; ODC, ornithine decarboxylase; TDA, tryptophan deaminase.

[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

they showed that certain marine bacteria were able to retain viability for as long as 70 weeks in conditions of starvation with no decrease in numbers. In support of the dormancy model proposed by Stevenson (20), many workers (11, 12, 21, 23) have suggested that marine bacteria retain viability during periods of nutrient stress by maintenance of a dormant state. Considering the characteristics of the ultramicrobacteria with particular emphasis on their response to nutrients, it seems likely that they may represent bacteria in such a state of dormancy. The only alternative which readily suggests itself is the possibility that the ultramicrobacteria represent a totally new class of bacteria. However, evidence reported by other investigators, as well as that presented here, does not favor such a conclusion. There is sufficient indication that the Perdido Bay isolates, rather than representing a new class of organisms, are marine bacteria of identifiable taxa which have expressed the ability to adapt to low nutrient levels.

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