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# Microcultural Study of Bacterial Size Changes and Microcolony and Ultramicrocolony Formation by Heterotrophic Bacteria in Seawater†

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With a microculture technique and time-lapse, phase-contrast photomicrography, it was possible to follow the division of individual cells and the development of microcolonies of bacteria in freshly collected marine water samples. A certain number of marine bacteria, upon inoculation onto a nutrient rich agar surface, displayed an increase in size as well as a high growth rate. Other bacteria were identified as very small marine bacteria (ultramicrobacteria). These had a very slow growth rate when inoculated onto a nutrient-rich agar surface. These latter cells formed very small microcolonies (ultramicrocolonies), and cell size did not increase significantly. These two types of marine heterotrophs could be described in terms of zymogenous and autochthonous bacteria, a concept used by Winogradsky for describing soil microorganisms.

In recent years there has been an increased interest in the microscopical observations of the morphology and size of marine bacteria present in freshly collected seawater. Direct microscopy (7, 8) and epifluorescent and phase-contrast microscopic techniques (4, 6, 10–13, 19, 20, 22, 23) have already confirmed the existence of very small coccoid forms (less than 0.3-μm diameter) in the sea, and these organisms may constitute a significant fraction of the bacterioplankton of the sea. For the purpose of this paper we have designated them as ultramicrobacteria. The term "minibacteria" was not used because of the possibility of confusing it with the minicells of *Escherichia coli* (1, 2) which are anucleated cells. The term "ultramicrocolony" has been used to describe the colonies formed by the ultramicrobacteria. These colonies cannot be seen with the naked eye. "Microcolony" is the term used to describe a small colony which may just be visible with the naked eye.

The ultramicrobacteria usually appear as coccoid cells when viewed through an optical microscope, but with transmission and electron microscopy the ultramicrobacteria appear not only as cocci but as vibrios, bacilli, horseshoes, and sigmoid forms (P. W. Johnson and J. M. Sieburth, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, N95, p. 178; Torrella and Morita, unpublished data). To the best of our knowledge none of the ultramicrobacteria have been cultivated and studied in pure culture, except for those formed by nutrient starvation in the laboratory (13).

When heterotrophic marine bacteria in seawater are cultivated in rich medium in vitro the average size of the cells is much larger than reported for marine bacteria freshly collected from the ocean and observed with epifluorescence or phase-contrast microscopy. This increase in size has been reported previously (8, 11). Nevertheless, a direct and clear optical demonstration of this fact at the level of the individual cell does not exist in the marine microbiology literature. No one experiment has yet shown how a freshly collected marine bacterial cell increases in size in response to laboratory cultural conditions. In this context various questions arise. What is the actual size of the easily cultured heterotrophs? Are these heterotrophs transient members of the bacterial population? Is their size in situ similar to the size they show on culture plates? What, if any, response do the ultramicrobacteria have when they come in contact with nutrient-rich medium? Since no microscopic evidence exists that demonstrates colony formation from ultramicrobacteria on agar surfaces, can this be demonstrated by a microcultural technique? To answer the foregoing questions, we have used microcultural methods and time-lapse photography.

## MATERIALS AND METHODS

**Sampling sites.** Two areas of Yaquina Bay (Newport, Oreg.) were sampled for this study. Seawater from the end of the Oregon State University Marine Science Center pier and from the Bay Front was collected in clear, sterile glass bottles and processed

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within 20 min as described below. The pier samples represented a non-contaminated site of the bay, whereas the samples from the Bay Front represented artificially enriched water. The latter receives some waste waters from fish and shrimp processing plants.

**Sample processing.** To find individual cells in the microscopic field for our microculture technique, the bacteria in the seawater samples had to be concentrated. The filtration of a known volume of seawater (200 to 250 ml of pier water or 50 ml of Bay Front water) through a Nucleopore polycarbonate membrane filter (0.2-$\mu$m pore size) was found to be the best method. Particulates on the filter were then suspended in a small volume (3 to 5 ml) of the same seawater. The yield of particulates was substantially increased by brushing the surface of the filter with a prewashed, sterile, small soft paint brush (camel hair or similar material). A heavy turbid suspension of particulates was obtained and used immediately for the microculture technique. Other methods of concentration, such as high-speed centrifugation, were time consuming and gave a substantially lower recovery of particulates—especially for ultramicrobacteria.

**Medium and incubation temperature.** Half-strength Lib-X medium was employed throughout this study. Lib-X has the following composition: yeast extract (Difco Laboratories), 1.2 g; Trypticase (BBL Microbiology Systems), 2.3 g; sodium citrate, 0.3 g; L-glutamic acid, 0.3 g; sodium nitrate, 0.05 g; iron sulfite, 0.005 g; Rila Marine Mix (salts), 35 g; and distilled water, 1 liter. The medium was adjusted to pH 7.8 and autoclaved. Although the foregoing ingredients were used at half strength, 12 g of agar (Difco) was used as a solidifying agent. Incubation was usually at room temperature (20 to 21°C), but in some cases 8 to 10°C was used. The in situ temperature of the seawater of Yaquina Bay during this investigation oscillated between 10 to 12°C.

**Microculture technique.** A block (approximately 1.5 by 1.5 cm) of agar medium was cut with a sterile spatula from a petri dish that had been previously filled with the culture medium to a depth of 2 mm. The filled petri plates had been left at room temperature for 2 to 3 days to obtain a relative dry surface and to allow oxygen to diffuse into the agar. The agar block was placed on the clean surface of a microscope slide and equilibrated at the desired temperature, and the agar surface was inoculated with 0.05 ml of the concentrated suspension of particulates. The suspension was spread over the surface with a small sterile bent glass rod. The partial dryness of the agar block allowed the absorption of the water and the fixation of the cells to the surface. The surface was then covered with a clean sterile cover slip and incubated at the desired temperature inside a petri dish on top of a piece of moist filter paper. The above procedure results in a very thin, water-saturated interface between the agar block and the cover slip. Two-dimensional growth occurs at the interface during the first divisional stages of the cell. Flagellated motile cells are not able to spread through the interface and remain in close proximity, except in the areas that surround big pieces of debris (e.g., fragments of diatoms) where motile cells move in the liquid phase. Cells with gliding motility can spread in the interface, but their movement is very slow and does not usually disturb the development of micro- and ultramicrocolonies.

**Microphotography.** A Leitz Ortholux microscope with an automatic photographic attachment (Leitz Orthomat), ×70 immersion oil phase-contrast objective (tube magnification factor, 1.25) and a Heine type phase-contrast condenser was used throughout this study. With this optical system it was possible to obtain good phase-contrast images of the particles in the interface of the agar block and the cover slip. A Kodak high contrast film (60 ASA) with a 12 ASA or lower setting in the Leitz Orthomat was used. A green filter was also employed to improve the contrast.

By using the calibrated microscope stage, 20 microscopic fields were randomly chosen and photographed at zero time. A simplified sketch of the distribution of particulates in each field chosen with the coordinates of the calibrated microscope stage helped identify the same fields so as to take pictures at various intervals of time. Between periods of microscopic examination the microculture slides were incubated at the desired temperature.

Although great care was taken in the focusing process, the edges of the cells are not as sharp as they appear to the naked eye due to the inherent difficulties in the resolution of the microscope. These ultramicrobacteria may appear a little larger than they should be. The blurred edges of these small cells not only made it difficult to determine the actual size but also did not permit us to distinguish clearly the differences in morphology between small rods, vibrios, horseshoe shapes, etc.

## RESULTS

Figure 1A to E shows the increase in size of a freshly inoculated bacterium during the process of cellular division. Figure 1F illustrates a portion of the microcolony at a stage in which hundreds of cells (not all shown) are present. The average size of the cells shown in Fig. 1F had decreased compared with those shown in Fig. 1C to E, but was still substantially larger than it was originally in freshly collected seawater (Fig. 1A). The pattern of size increases and decreases in Fig. 1 paralleled closely the lag phase (Fig. 1A to C), initial exponential phase (Fig. 1D and E), and exponential phase (Fig. 1F) of growth.

Figure 2 is another clear example of the response of bacteria placed onto a rich medium. The cell(s) labeled no. 1 increased in size quite noticeably. As the microcolony grew, the cell size became more or less stabilized.

Strange bacillary to vibrio-like cell morphologies can be seen in Fig. 2B to E. Such bizarre large cells were also observed in parallel experiments with known marine *Vibrio* and *Pseudomonas* species as the inoculum in the microculture technique. These somewhat bizarre forms can also be observed in the lag phase of batch cultures when heterotrophic bacteria are cul-

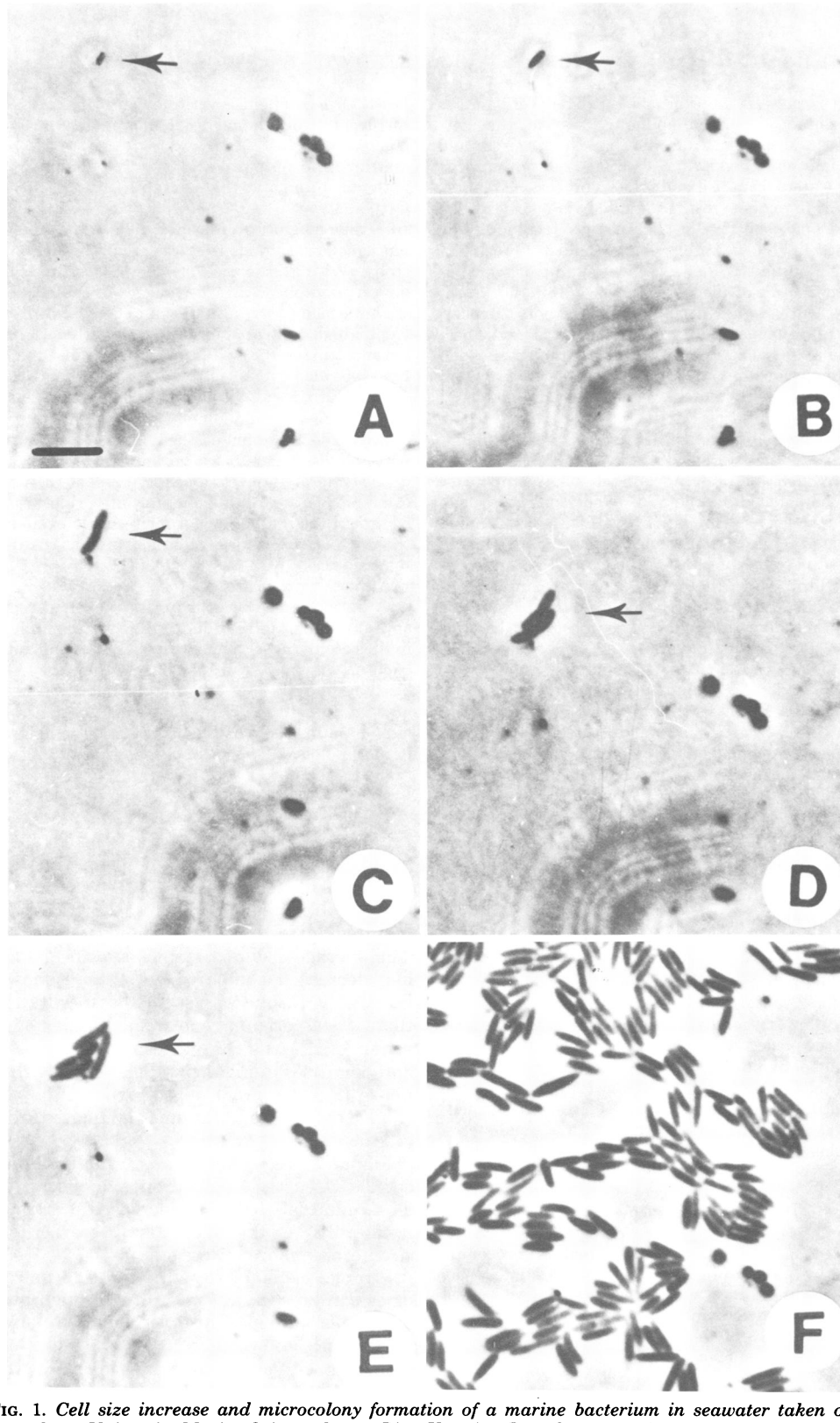


FIG. 1. *Cell size increase and microcolony formation of a marine bacterium in seawater taken off the Oregon State University Marine Science Center Pier, Yaquina Bay, Oreg., and incubated onto a Lib-X agar surface by the microculture technique. Incubation temperature was 20 to 21°C; A, 0 h; B, 3 h; C, 6 h; D, 7 h 20 min; E, 9 h 10 min; and F, 19 h. Bar, 5 μm. The dispersion of cells in F is typical of bacteria capable of gliding at the interface.*

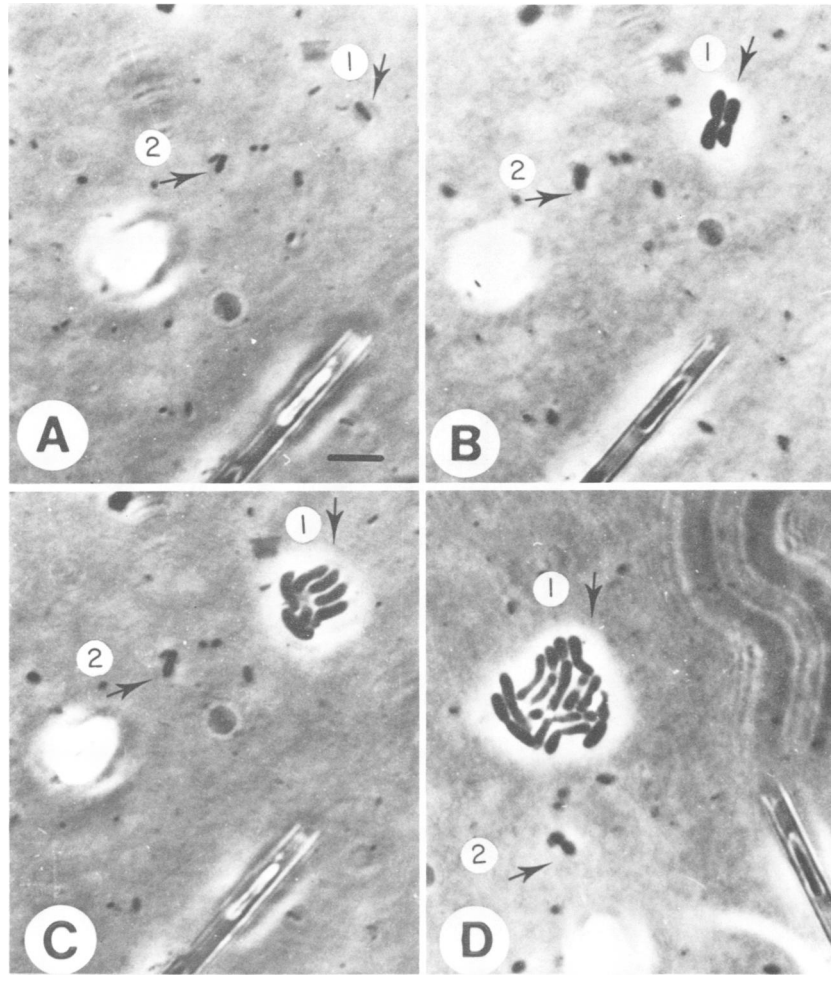


FIG. 2. *Cell size increase and microcolony formation of marine bacteria in seawater taken off the Oregon State University Marine Science Center Pier and incubated onto a Lib-X agar surface by the microculture technique. Incubation temperature was 20 to 21°C; A, 0 h; B, 3 h 45 min; C, 5 h; D, 6 h 15 min; E, 8 h 15 min; F, 12 h 15 min; G, 30 h. Bar, 5 μm. A piece of a marine diatom can be seen at the edge of the photomicrographs. Cell no. 2 completed only one division before being engulfed by the growing microcolony.*

tured in rich organic medium. Figure 2 also shows another interesting observation present in a number of our microculture slides. The cell labeled no. 2 was only able to make one division accompanied by an increase in cell size.

Figure 3 is a sequence of photos of microcultural slides inoculated with Bay Front water which is enriched with wastewater from shrimp and fish processing plants. Cells were large, as indicated in the zero time photo, and there did not appear to be a detectable increase in cell size with time. This situation did not appear to be common in the bay. Bacteria collected from other relatively rich habitats, such as algal mats, did increase their cell size when inoculated onto the nutrient-rich medium used in our microculture technique. When the time interval between sequences of photos of Fig. 3 is compared with Fig. 1 and 2, the lag period appears to be shorter in the former, an indication that the cells may have already physiologically adapted to growth in a nutrient-rich habitat.

The cells shown in Fig. 1, 2, and 3 that increased in size and formed microcolonies are probably typical of the type of bacteria grown on nutrient agar surfaces to form visible colonies. However, the microculture technique permitted the observation of another group of very small marine bacteria (ultramicrobacteria). These ultramicrobacteria, when they were divided, formed very small microcolonies (Fig. 4 to 7), which we have termed ultramicrocolonies for

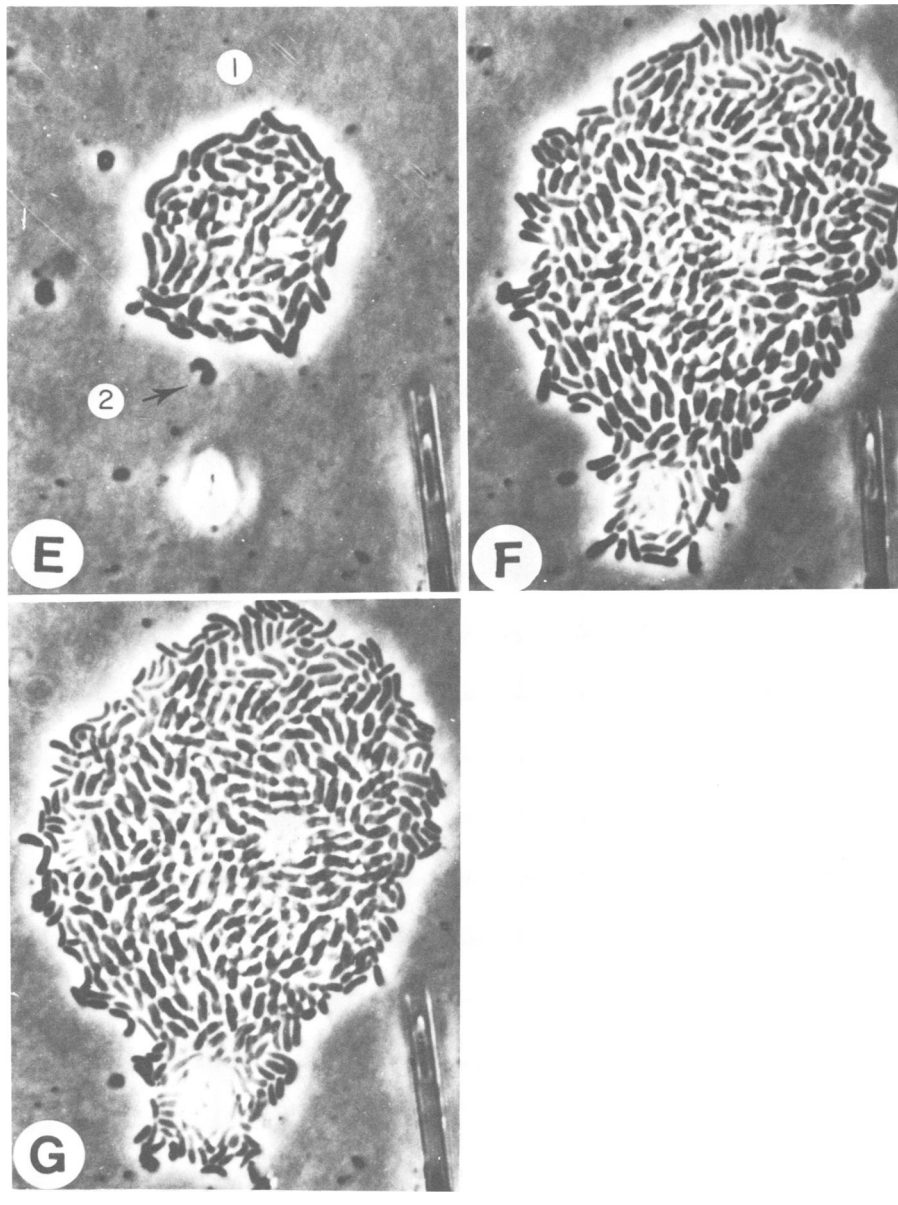


FIG. 2E–G.

the purpose of this paper. Ultramicrocolonies never reached a significant size and were usually pushed aside by the progressive growth of the microcolonies of the fast-growing heterotrophs (Fig. 5). Other ultramicrobacteria increased their size slightly before and after division but remained small. The lack of a significant increase in size could be correlated with a slow growth rate on the rich medium employed.

Figure 4 shows the edge of a microcolony of a fast-growing heterotroph approaching an ultramicrocolony. Around the ultramicrocolony other bacteria have undergone just a few divisions. The size difference in both cells and colonies are very evident in Fig. 5, which also shows how a microcolony of a fast-growing heterotroph crowds an ultramicrocolony resulting in ultramicrobacteria becoming intermingled with the large cells.

Figure 6 shows how a colony of a fast-growing

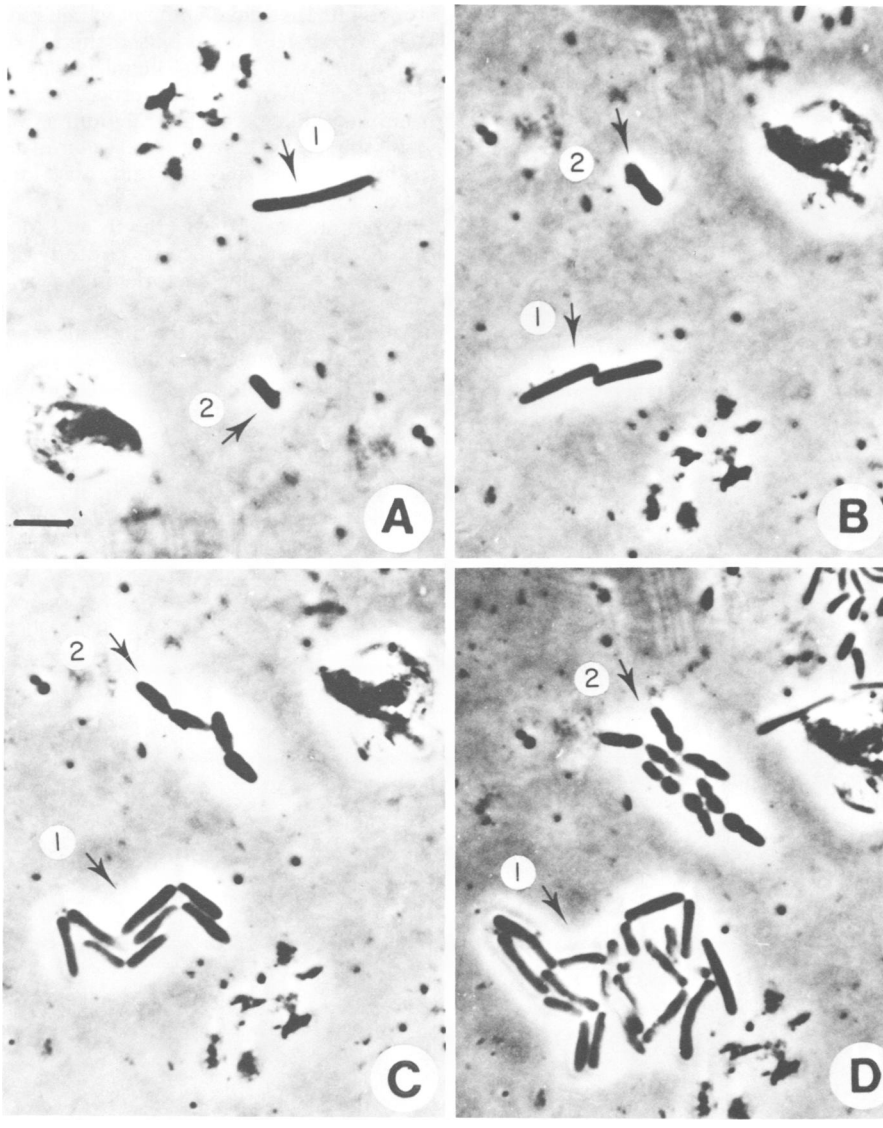


FIG. 3. *Microcolony formation of marine bacteria in seawater taken off the Bay Front, Yaquina Bay, Oreg., A, 0 h; B, 1 h; C, 3 h 45 min; D, 5 h 45 min. Incubation was 20 to 21°C. Bar, 5 μm. Cell size did not increase in the process of microcolony formation, mainly because the water sample had been enriched with organic matter.*

species invaded an area where two ultramicrocolonies had developed. Fig. 6B and C are separated by only a 30-min interval. During this time the big colony advanced significantly relative to the piece of debris in the field. This progression implied many cell divisions and was a direct indication that cells were actively growing. On the other hand, the ultramicrocolonies labeled no. 1 and 2 had reached the stage of having only 12 and 5 cells, respectively, after 19 h of incubation and did not divide any further in the next 20 min.

In the series of photos in Fig. 7 different examples of the growth pattern of ultramicrobacteria are shown. Cell no. 1 was able to form an ultramicrocolony that did not grow any further after 18 h of incubation. The size of the individual cells remained small. Cell no. 2 was able to divide only once during the 25 h of incubation. Cell no. 3 was able to increase in

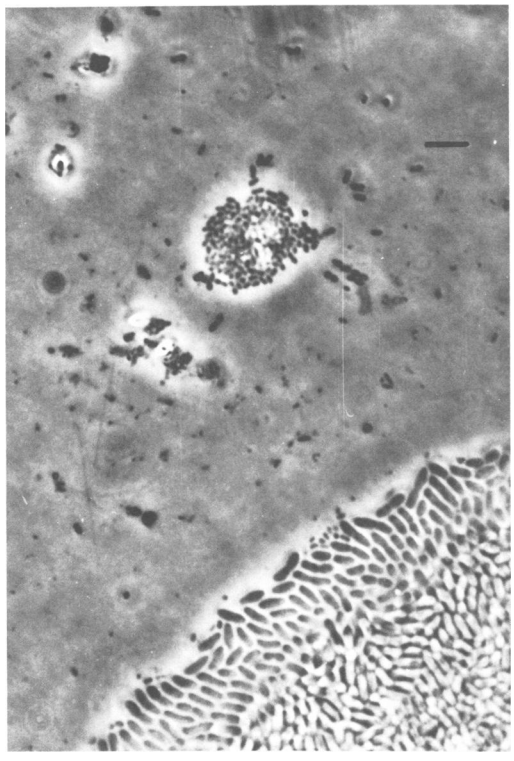

FIG. 4. *Photomicrograph of a microculture slide of bacterial cells in seawater collected from Yaquina Bay and incubated for 24 h. An ultramicrocolony can be seen in the center of the photo, and the edge of a microcolony of a fast-growing heterotroph can be seen at the bottom right. Incubation temperature was 20 to 21°C. Bar, 5 μm.*

size, but never divided, whereas cell no. 4 formed a five-celled ultramicrocolony.

## DISCUSSION

We recognize drawbacks of the technique used, such as nutrient exhaustion, harmful excreted compounds of metabolism, oxygen supply, substrate accelerated death, etc. Furthermore, we recognize that the microculture technique does not offer to the freshly collected marine bacteria a habitat similar to the situation in situ. However, we are confident that the conditions in the microculture technique are similar to the situation in which bacterial cells are inoculated onto a nutrient-rich agar surface for isolation purposes. Using a known marine *Vibrio* species under identical conditions, we observed that microcolonies did not stop their growth and eventually became visible to the naked eye, as one would expect from a facultative anaerobe. With *Pseudomonas* species the microcolony stopped further development, which indicated a lack of oxygen. We have used the microculture technique to investigate the initial development of microcolony and ultramicrocolony formation from individual cells on a medium as well as to view the size difference between bacteria in freshly collected seawater and those on a nutrient agar surface.

Based on the rate of growth and increase in cell size in our microcultural study, we have divided the marine bacteria into two distinct types. The first type comprises cells that are able to quickly colonize the nutrient agar surface as a result of their fast growth rate, and the second type comprises the ultramicrobacteria which are characterized by a slow growth rate. Both types are based on the Lib-X medium employed in this study.

The first type is typical of the marine heterotrophic bacteria generally isolated and studied by investigators. These bacteria grow on 2216

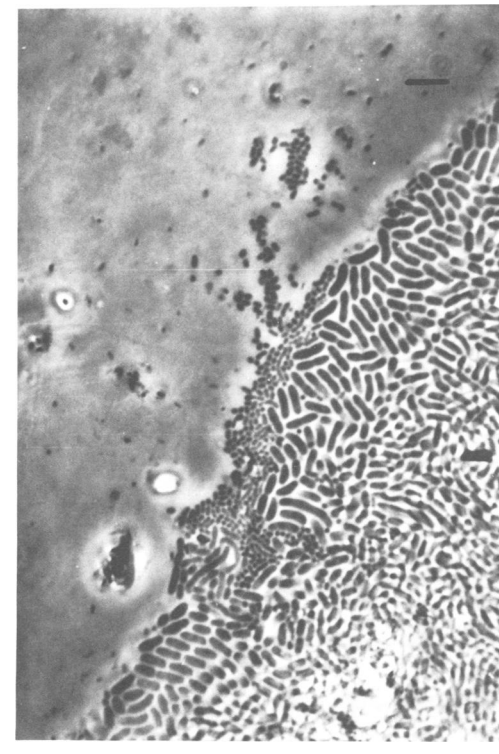

FIG. 5. *Photomicrograph of the contact between an ultramicrocolony and a microcolony on a microculture slide inoculated with bacteria from seawater collected off the Oregon State University Marine Science Center Pier. Incubation temperature was 20 to 21°C. Bar, 5 μm. The microcolony (right) is pushing the ultramicrocolony, whose structure has been disrupted in the process.*

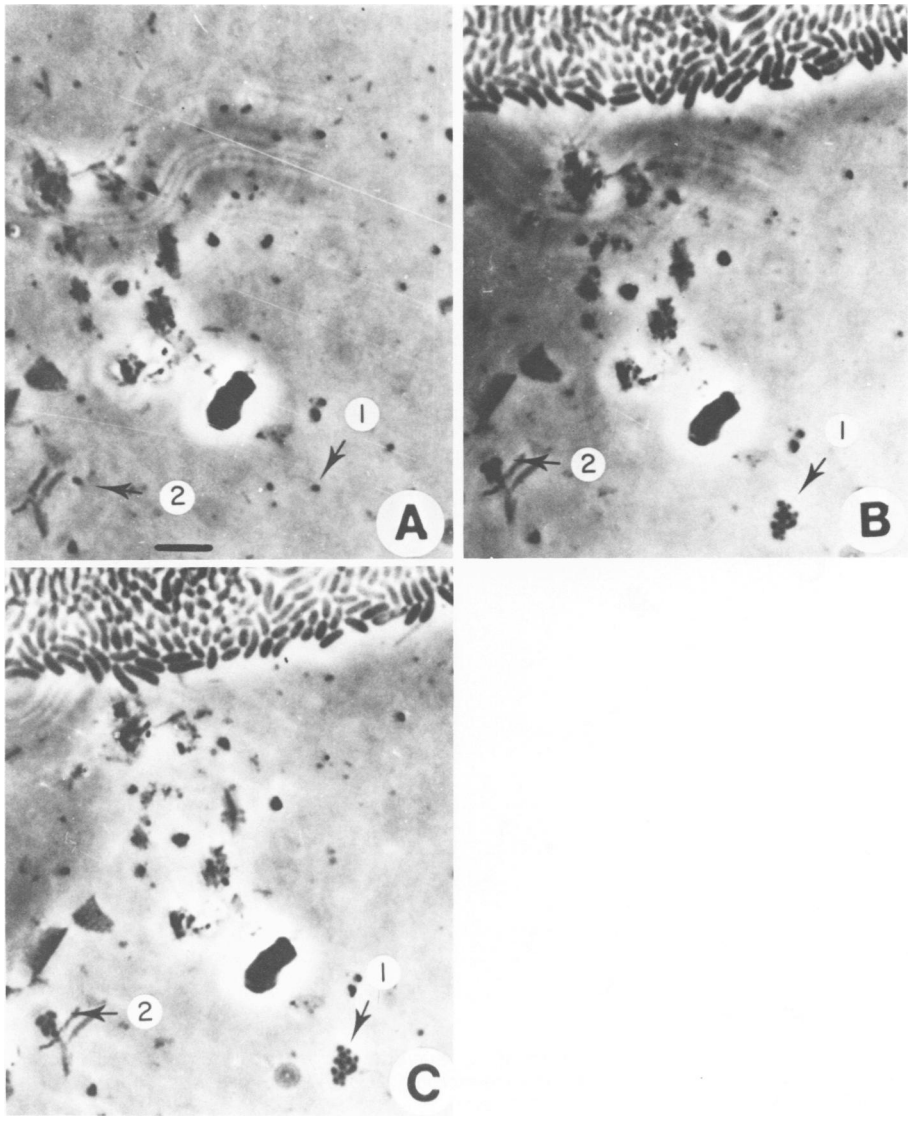


FIG. 6. *Photomicrographs illustrating the development of a microcolony and two ultramicrocolonies, A, 0 h; B, 19 h; C, 19 h 30 min. Incubation temperature was 20 to 21°C. Bar, 5 μm. Colony no. 2 moved a little to the left of a piece of debris in B and C compared with A.*

agar (Difco) or some other nutritive agar medium, and the assumption is commonly made that they can grow in seawater. Many investigators, such as Donachie (5), Helmstetter et al. (9), and Paau et al. (14), have described an increase in size of the bacteria in lag and exponential phases. An increase in size is also noted in bacteria and yeasts when growth rates are increased (9, 10, 14, 16, 17), a phenomenon known as nutrient modulation (10). Figures 1 and 2 are representative of this rapidly growing type of bacteria. According to Winogradsky's classification (21) and the suggestion of Jannasch (7, 8), these organisms could be classified as zymogenic and would only grow rapidly and increase in size when energy was available in the ocean (e.g., decaying fish), but when these organisms were in nutrient-poor seawater they were in a state of low metabolic activity or dormant; hence, they were smaller. An exception to this in our study would be the cells shown in Fig. 3, since they did not increase in size because the natural situation probably supported a high growth rate. Because of the low nutrient content

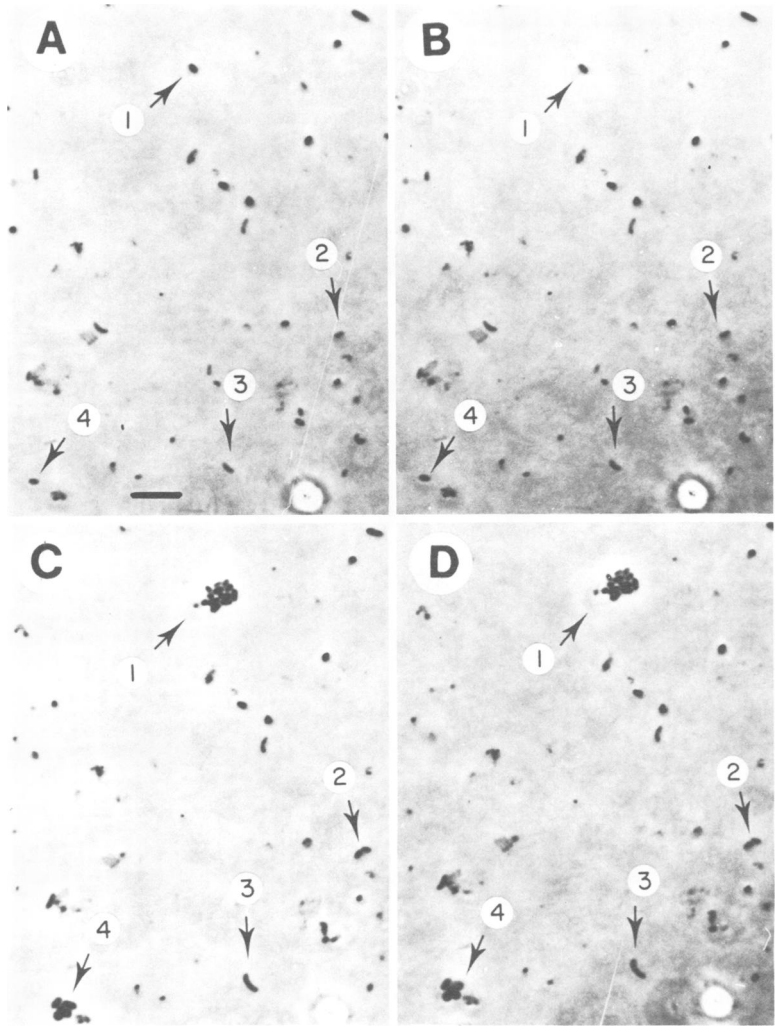


FIG. 7. *Photomicrographs of a microculture slide, inoculated with water from a clean area of Yaquina Bay, Oreg., illustrating the growth pattern of ultramicrobacteria; A, 0 h; B, 2 h; C, 18 h 15 min; D, 25 h 30 min. Bar, 5 μm. Incubation temperature was 20 to 21°C.*

of seawater, a low metabolic rate or a state of dormancy probably exists (18).

The second type of heterotrophic bacteria (ultramicrobacteria) (Fig. 4 to 7) display a slow growth rate even on nutrient medium and do not increase in size before or during ultramicrocolony formation. These may be the autochthonous microbial populations of the seawater sample and may include bacteria that have adapted to the low concentrations of organic matter in seawater. In the presence of a high concentration of organic matter, as represented by the nutrient agar, they still keep their low metabolic and growth rates and, as a result, form ultramicrocolonies.

All of the photomicrographs presented in Fig. 1 to 7 were from microcultures incubated at 20 to 21°C. This represents a temperature of 10 to 12°C above the water temperature of the Bay during the sampling period. At temperatures of 8 to 10°C, there was a clear decrease in the rate of colony formation by the fast-growing heterotrophs, as would be expected. Cell size increase was again noted at the low temperature. Energy availability appeared to be the key to the increase in cell size of the fast-growing heterotrophs; energy availability is also evident with the cells from waters rich in organic matter (Fig. 3). Shehata and Marr (17) stress the importance of nutrients as well as the growth rate on the mean cell size of nonspecific bacterial populations.

Cell no. 2 in Fig. 2 only made one division during the experiment. A number of possibilities might account for this behavior: (i) the interface was physically inappropriate for growth of the organism, (ii) chemical and physical conditions were wrong, (iii) some antimetabolite excreted by other cells that inhibited cell no. 2, (iv) death was accelerated by the substrate, or (v) there was some harmful interaction among microcolonies. The cessation of growth of the ultramicrocolonies in Fig. 6 is harder to explain since a microcolony of fast-growing heterotrophs is not in close proximity.

We recognize that there are more sophisticated microculture techniques (15) that could be used in this type of study that make use of medium composition, incubation temperature, etc. However, it is hoped that the presentation of the ultramicrobacteria and the development of ultramicrocolonies will induce investigators to look more closely at them and assess their importance in the marine environment. If the ultramicrobacteria presented here are the autochthonous bacteria in the seawater, then they may be more important in the ecosystem than the zymogenous ones which probably do not have enough energy to take care of their own growth needs at all times. Carlucci and Shimp (3) noted that some marine bacteria could grow at low substrate concentrations, but their bacteria designated as 150 could also grow to a density of $10^9$ cells per ml when supplied with 0.5 to 5.0 g of peptone per liter. In other words, the zymogenic bacteria often become deprived of an energy source under natural conditions. Because the ultramicrobacteria are probably not isolated on nutrient-rich media, they have been neglected by investigators. Their possible importance should not be overlooked—mainly because they may not require a high concentration of nutrients to carry on their metabolic processes.

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