Changes in Cellular States of the Marine Bacterium *Deleya aquamarina* under Starvation Conditions

FABIEN JOUX,¹ PHILIPPE LEBARON,^{1*} AND MARC TROUSSELLIER²

Observatoire Océanologique, Université Paris VI, Institut National des Sciences de l'Univers,
URA CNRS 2071, Banyuls-sur-Mer Cedex,¹ and Laboratoire d'Hydrobiologie Marine
et Continentale, Université de Montpellier II, UMR CNRS 5556,
F-34095 Montpellier Cedex 05,² France

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In this study, we have used different fluorescent dyes and techniques to characterize the heterogeneity and changes of the physiological states encountered by the marine bacterium *Deleya aquamarina* during a 92-day starvation survival experiment at 20 and 5°C. Changes of physiological states were investigated on a single-cell basis by flow cytometry and epifluorescence microscopy in conjunction with fluorescent dyes specific for various cellular functions and constituents. Heterogeneities within populations with regard to functions (respiration, substrate responsiveness, enzymatic activity, and cytoplasmic membrane permeability), constituent (DNA), and cell volume (light scatter) were compared to the evolution of viable plate counts (CFU). At 20°C, CFU changes were divided into three stages corresponding to stability up to day 13 followed by a rapid drop between days 13 and 42 and then by stabilization at a level of 10 to 20% during the remaining survival period. Most of the cellular fractions showing a metabolic activity were close to the evolution of the culturable cells, suggesting the absence of viable but nonculturable cells. On the other hand, cells with selective cytoplasmic membrane permeability but without any metabolic activity were observed, and this stage was followed by DNA alteration occurring at different rates after the loss of membrane cytoplasmic permeability. We observed a greater maintenance of culturability, physiological functions, DNA, and cellular volume at the lower temperature. These results have different ecological implications from both methodological and conceptual viewpoints.

Starvation is the most common mode of bacteria in marine ecosystems. The amount of dissolved organic matter in the oceans is between 0.2 and 2.0 mg of carbon/liter, and most of it is present in a polymeric form (i.e., not readily utilizable for microbial growth) and/or not biodegradable (35). The low concentrations of organic and inorganic matter added to the heterogeneously distributed nutrient sources may be responsible for an important distribution of bacterial populations in terms of activity, leading to the existence of growing, active, nonactive, and dead cells (55). Characterization of the physiological state of individual cells is of great importance in understanding the functioning of microbial communities (11) as well as the kind of survival strategies that may be used by organisms to face their environment.

The recent development of fluorescent dyes and methods, offering probes for a variety of cellular functions and constituents, and flow cytometry (FCM) techniques has made the characterization of the physiological states of bacterial individual cells possible (for reviews, see references 34 and 44). Some of these methods have been applied to natural systems (9, 11, 25, 45, 48, 56). However, their application to complex natural communities is often biased since most of them depend on the type of organisms within communities and on the chemical environment of the cells. For instance, the direct-viable-count (DVC) method (25) is biased by the different sensitivities of organisms to antibiotics and to the short incubation time, which is limited by the growth of some resistant and active cells. Similarly, respiratory measurements may be biased by the

chemical composition of natural samples (47) as well as by the existence of different respiring pathways (57, 62). When applied to natural communities, the interpretation of these physiological measurements and their significance in terms of the activity of individual cells remain unclear today.

As stated by Lenski (31), "simple laboratory systems cannot, of course, capture all the richness and complexity of natural ecological systems, however, such systems do permit careful analyses of population dynamic under well-defined conditions and moreover, rigorous experimental test of mechanistic hypotheses." The cellular analysis of bacterial physiology in autecological studies has led to an increasing understanding of the response of bacterial cells to environmental stresses. The most common cellular response to nutrient scarcity is the ability of bacteria to adopt a physiological state under which they cannot be grown by conventional culture methods but are able to retain certain assayable activities or physiological functions. This so-called viable but nonculturable (VBNC) state has been investigated mainly for some pathogenic bacteria (e.g., *Salmonella* spp., *Escherichia* spp., *Campylobacter jejuni*, and *Vibrio cholerae*) due to their public health implications. Recently, it was shown that *S. typhimurium* cells may undergo a succession of physiological states under stressful conditions (24). With a few exceptions (4, 9, 20, 26), studies of the physiological heterogeneity of marine bacteria under stressful conditions were addressed mainly to pathogenic estuarine *Vibrio* spp. (e.g., *V. vulnificus*) exposed to low temperature (see e.g., references 7, 13, 17, 22, 39, 41, 42, 50, 61, and 64). Further investigations of the behavior of some major groups of marine bacteria are needed to improve our understanding of the transition steps (i.e., the characterization of physiological states and importance of each of them) as well as the significance of cellular techniques to characterize bacterial activity.

In this work, we studied changes in the physiological state of

* Corresponding author. Mailing address: Observatoire Océanologique, Université Paris VI, Institut National des Sciences de l'Univers, URA CNRS 2071, BP44 F 66651, Banyuls-sur-Mer Cedex, France. Phone: (33) 2-68887353. Fax: (33) 2-68887395. E-mail: lebaron@arago.univ-perp.fr.

individual cells of the marine bacterium *Deleya aquamarina* (1). The genus *Deleya* is a common component of the culturable heterotrophic bacterial communities in seawater (6, 43). All *Deleya* species are strictly aerobic, gram-negative straight rods which require sodium ions for growth (1). Recently, phylogenetic studies have unified the genera *Deleya*, *Halomonas*, and *Halovibrio* into a single genus, *Halomonas* (12). Changes in the physiological states of the cells were investigated at the cellular level during 92-day nutrient starvation experiments. Experiments were performed in artificial seawater microcosms at two temperatures representative of the temperature range in the Mediterranean (5 and 20°C). We monitored changes in viable plate counts (CFU) and those of single cells presenting respiratory activity, substrate responsiveness (DVC), esterase activity, and cytoplasmic membrane permeability as determined by FCM and epifluorescence microscopy. Changes in cellular DNA content and light-scattering signal were also analyzed.

MATERIALS AND METHODS

Growth and starvation conditions. Stock cultures of *D. aquamarina* (Collection Institut Pasteur, C.I.P. 74.8T, Paris, France) were prepared from a log-phase culture and stored at -80°C in marine broth (MB; Difco) supplemented with glycerol (40%, vol/vol). When needed, 100 µl of the stock culture was added to 20 ml of MB and grown for 48 h at 20°C. Then, 1 ml was placed in a 250-ml flask containing 100 ml of MB and grown at 20°C with magnetic agitation. Under these growth conditions, the generation time was about 2 h. After 44 h of incubation, corresponding to the stationary phase, 40 ml was harvested by centrifugation at $7,000 \times g$ for 10 min at 18°C. The pellets were washed twice with artificial seawater (ASW; see below) and then resuspended in 10 ml of ASW.

Each survival assay was conducted in an acid-washed 1-liter Erlenmeyer flask containing 400 ml of ASW. ASW was prepared by dissolving 37.5 g of Sea Salts (Sigma) in 1 liter of distilled water supplemented with 10 mM (3-[N-morpholino]propanesulfonic acid) (MOPS; Sigma). The final solution was adjusted to pH 8.0 with concentrated NaOH and then filtered through a 0.45-µm-pore-size membrane (Millipore). The final salinity of the ASW was 3.5‰. The flasks were autoclaved (120°C for 15 min), cooled, inoculated to a cell density of approximately 10^7 cells per ml, and incubated in the dark at both 20 ± 0.5 and 5 ± 0.5 °C. The cells were kept in suspension by gentle stirring.

Total-cell and culturable-cell (CFU) enumerations. Samples for total-cell enumerations were fixed with formaldehyde to a final concentration of 2% (vol/vol), and stored at 4°C until used. After being stained with 4',6-diamidino-2-phenylindole (DAPI; 2.5 µg/ml) for 30 min and filtered through black polycarbonate membrane filters (pore size, 0.2 µm, 25-mm diameter; Nuclepore), the cells were counted with an Olympus BH-2 epifluorescence microscope (100-W mercury lamp; filter set U [UG-1, DM400, and L420]). For each sample, a minimum of 20 fields (≥ 600 cells) were counted and averaged (29).

For enumeration of culturable cells, 100-µl serial dilutions (1/10) in ASW were plated onto marine agar (Difco). For the starvation regimen at 5°C, dilution tubes and agar plates were held at 5°C before use. Colonies were counted after 48 h and 7 days of incubation at room temperature (± 20 °C). Prolonged incubation did not yield higher CFU counts. The proportion of culturable (CFU⁺) cells was defined as the ratio of the number of CFU⁺ cells to the total number of cells determined by DAPI staining.

FCM. FCM analyses were performed with an ACR 1400 (Bruker Spectrospin, Wisssembourg, France) flow cytometer equipped with a 100-W mercury lamp. Light-scattering and fluorescence parameters were acquired by using a logarithmic gain mode. Optical alignment was based on the optimized signal from fluorescent 1.97-µm-diameter microspheres (no. 09847; Polysciences Europe, Eppelheim, Germany). Fluorescent 0.94-µm microspheres (Polysciences no. 17154) were added to each sample as an internal fluorescence standard.

DNA content and light-scattering measurements. Formaldehyde-fixed samples were diluted (1/10, vol/vol) in phosphate-buffered saline (pH 7.2) and stained with 2.5 µg of Hoechst 33342 (HO342; Sigma) per ml for 2 h. FCM analyses were performed with an excitation wavelength of 360 to 370 nm. Emission was collected through a 400-nm dichroic filter, a 420-nm long-pass filter, and a 540-nm short-pass filter. The analysis of the cells was triggered on the blue fluorescence. Minimum gates on forward-angle light scatter (FALS) and blue fluorescence amplifiers were set to exclude subcellular debris and optical "noise."

Substrate responsiveness. FCM was used to enumerate substrate-responsive cells as determined by the DVC method (25). Substrate-responsive (DVC⁺) cells were characterized by a cellular elongation during incubation in the presence of nutrients and nalidixic acid, a specific inhibitor of DNA gyrase that prevents cell division but not metabolic activity. Nalidixic acid (Sigma) was dissolved in 0.05 N NaOH (stock solution, 2 mg/ml) and sterilized through 0.2-µm-pore-size membrane filters (Sartorius). A 1-ml volume of sample was added to 5 ml of diluted

MB (1/10, vol/vol) containing 20 µg of nalidixic acid per ml (final concentration), corresponding to the MIC for the strain. Then, samples were incubated in the dark at 20°C, fixed after 10 and 24 h by adding formaldehyde to a final concentration of 2% (vol/vol), and stored at 4°C. Control samples were fixed before incubation in the DVC medium. The cells were stained with DAPI (2.5 µg/ml) for 30 min and analyzed by FCM with the same configuration as for HO342 staining (see above). The data were displayed as dual-parameter FALS and wide-angle light-scattering (WALS) histograms. Windows circumscribing the nonelongated cells (control) (Fig. 1A) and the elongated (DVC⁺) cells (Fig. 1B) were set to enumerate the selected populations. The proportion of DVC⁺ cells was defined as the ratio of the number of elongated cells to the total number of cells (elongated cells plus nonelongated cells). When DVC⁻ and DVC⁺ areas overlapped (see Fig. 1B), the difference between cells recorded in the DVC⁺ area before and after incubation was considered the score of DVC⁺ cells. On day 23 of the experiment, it was necessary to increase the incubation period from 10 to 24 h to better discriminate elongated from nonelongated cells. By day 92, the antibiotic concentration was lowered from 20 to 10 µg/ml to maintain a similar effective cessation of cell division, because of an increase of cell sensitivity under starvation.

Respiratory activity. 5-Cyano-2,3-dimethyl tetrazolium chloride (CTC; Polysciences Europe) reduction was used to identify respiring cells by the method of Rodriguez et al. (52). CTC was added (5 mM, final concentration) to diluted samples (1/10, vol/vol) in saline buffer (NaCl, 35 g/liter; MOPS, 20 mM [pH 8]). CTC incubations were performed in the dark at the microcosm incubation temperatures (20 and 5°C). After incubation, the samples were fixed with 2% formaldehyde (final concentration) and stored at 4°C until enumeration. The cells were then counterstained with DAPI (2.5 µg/ml) for 30 min and filtered through black polycarbonate membrane filters (pore size, 0.2 µm, 25-mm diameter; Nuclepore). Respiring (CTC⁺) cells were enumerated by epifluorescence microscopy with filter set B (BP490, DM500, and AFC+O515), and then total cells were counted by switching to filter set U (UG-1, DM400, and L420). A minimum of 400 cells were counted for each sample. The control sample was treated with formaldehyde (2%, vol/vol) before incubation with CTC. The proportion of CTC⁺ cells was calculated as the ratio of CTC⁺ cells to total cells (DAPI⁺ cells).

Enzymatic activity. Intracellular enzyme activity was assessed with the fluorogenic substrate Chemchrom B (Chemunex, Maisons-Alfort, France). This non-fluorescent dye is transported across the bacterial membrane and cleaved by intracellular nonspecific esterases (supplier information), releasing a fluorescent product. Product retention indicates expression of cytoplasmic enzymes and also membrane integrity. Chemchrom B (10 µl) was added to 1 ml of diluted samples (1/10, vol/vol) in the reaction buffer provided with the kit. Incubation was carried out in the dark at room temperature for 30 min. After labeling, samples were kept in the dark on ice for at least 10 min. The use of the blocking reagent (Chem red) supplied with the kit was not necessary. FCM analysis used an excitation wavelength of 470 to 490 nm and an emission wavelength of 540 ± 20 nm. Heat-killed cells (60°C for 1 h) were used as a control for FCM determination of positive cells (Fig. 2A).

Cytoplasmic membrane permeability. Cytoplasmic membrane permeability was assayed by FCM with the Live/Dead BacLight kit (Molecular Probes Europe, Leiden, The Netherlands). The kit contains two nucleic acid stains differing both in their spectral characteristics and in their ability to penetrate healthy bacterial cell membranes (supplier information). A mixing of the two reagents was prepared (1/1, vol/vol) and added (2/1,000, vol/vol) to samples previously diluted in ASW (1/10, vol/vol). The samples were incubated for 15 min at room temperature in the dark and analyzed by FCM. FCM analysis used an excitation wavelength of 470 to 490 nm. Green and red fluorescence were separated with a 560-nm dichroic filter and respectively selected by 540 $\pm$ 20-nm and 590-nm long-pass plus 720-nm short-pass filters. The control was obtained by ethanol fixation (70%, vol/vol) before staining. The data were displayed as dual-parameter histograms of green fluorescence/red fluorescence intensities. Figure 3A shows a population of *D. aquamarina* cells treated by ethanol to permeabilize the cytoplasmic membrane. These cells show reduced green and red fluorescence compared with untreated cells on day 0 of survival (Fig. 3B). Windows circumscribing the cells with a damaged cytoplasmic membrane (MEMB⁻ cells as determined by ethanol-fixed sample) and the cells with an intact cytoplasmic membrane (MEMB⁺ cells) were set to determine the cell abundances of selected populations. The proportion of MEMB⁺ cells was determined by dividing the MEMB⁺ cell number by the total cell number (MEMB⁺ cells added to MEMB⁻ cells).

Reproducibility of results. Duplicate experiments were performed at both temperatures. For those at 20°C, the results of duplicate experiments are presented separately due to the existence of slight variations for some parameters. For other results, the mean values from duplicate experiments and standard deviation are reported in Results. Each cellular parameter used in this study was tested for reproducibility. In all cases, triplicate measurements show a coefficient of variation of <5%, except for total and culturable cell counts, which show a coefficient of variation of about 10%.

FIG. 1. FCM analysis of DVC. The x axis shows FALS, and the y axis shows WALS. The distribution of untreated cells at the onset of starvation (control, nonelongated cells) (A) and the distribution after DVC incubation of cells after 0 days (B), 22 days (C), and 92 days (D) of starvation at 20°C are shown.

RESULTS

Changes during survival at 20°C. For both experiments, total cell counts increased slightly (approximately twofold) during the first days of starvation and then differed between replicate experiments (Fig. 4A). In the first microcosm, total counts were constant throughout the entire starvation period, whereas in the second, they decreased slightly from 2.4×10^7 (day 4) to 9.2×10^6 (day 92) cells per ml. In both experiments, the CFU curve could be divided into three stages. During stage 1 (0 to 13 days), the percentage of culturable cells remained relatively stable and close to 100%; stage 2 (13 to 42 days) corresponded to a rapid decrease in the culturable cell fraction; and stage 3 (35 to 92 days) was characterized by the stabilization of the percentage of culturable cells representing 10 and 20% for experiments 1 and 2, respectively.

The evolution of the different physiological cell categories is shown in Fig. 4B. Under starvation at 20°C, most of the cells were responsive to the different assays from the onset of starvation and during stage 1. Stage 2 corresponded to a concomitant decrease of the DVC⁺, CTC⁺, and ENZ⁺ fractions and a stability of the MEMB⁺ fraction. During stage 3, the DVC⁺, CTC⁺, and ENZ⁺ fractions stabilized at approximately 10% of the total population in experiment 1 but increased slightly (~10%) in experiment 2. This different behavior at the end of the two experiments may be explained by the fact that some cell lysis occurred more rapidly in experiment 2, increasing the relative cell fractions of cells showing metabolic activity. For both experiments, the MEMB⁺ fraction showed a large decrease in the first part of stage 3 (from days 35 to 72) and

then stabilized at the level of other cell fractions. Discrimination during starvation between positive and negative subpopulations with respect to the different physiological functions is illustrated in Fig. 1C and D for substrate responsiveness, Fig. 2C and D for enzymatic activity, and Fig. 3C and D for membrane permeability.

During stage 1, FALS measurements indicated a rapid decrease related to a reduction of the cellular volume as observed by microscopy, and no further reduction was found during the following stages (Fig. 4C). The relative DNA content per cell analyzed by FCM showed differences between the two experiments (Fig. 4C). The DNA content did not change during experiment 1 but was subjected to a regular decrease during stages 2 and 3 in the other experiment. The recorded DNA fluorescence also indicated modification in cell genomic distribution (Fig. 5). At the onset of starvation, the broad DNA distribution indicates a continuum of DNA contents without resolved peaks, suggesting that residual cell growth occurred even during the stationary phase (Fig. 5A). On day 4 of starvation, the DNA distribution showed well-defined peaks, corresponding to cells with different genomic contents. This distribution remained unchanged for both experiments until 13 days of starvation, with 13.5, 60.5, and 26% of cells containing one, two and four genomes, respectively (Fig. 5B). During stages 2 and 3, the DNA distribution was slightly modified in terms of peak resolution in experiment 1 (Fig. 5C), while a degradation of cell fluorescence was observed for a fraction of the population in experiment 2, with an increased number of cells containing one genome (Fig. 5D).

FIG. 2. FCM analysis of esterase enzymatic activity. The x axis shows green fluorescence, and the y axis shows the relative cell numbers. Results for heat-treated cells (control) (A) and cells after 0 days (B), 35 days (C), and 92 days (D) of starvation at 20°C are shown.

Changes during survival at 5°C. When incubation was performed at 5°C, the total counts remained constant, indicating no lysis of these cells (data not shown). Comparisons between the average percentages of physiological categories during survival at 20 and 5°C are presented in Table 1. The standard deviations were determined between the two replicated microcosms to show the lower reproducibility of all parameters recorded at 20°C and the homogeneity of the results at 5°C. Enzymatic activity was not reported for starvation at 5°C, because discrimination between positive and negative cells was unclear and it was not possible to define cellular categories (data not shown). This may be due to changes in membrane permeability to the Chemchrom B substrate or an enzymatic inactivation at low temperature. A high percentage ($\geq 70\%$) of culturable cells was maintained throughout the starvation period at 5°C. For all samples, the percentage of cells showing an active physiological function was higher or equal at 5°C than that recorded at 20°C. At the lower temperature, cells showed minor reduction in FALS values, while the cell cycle regulation at the early stage of starvation was similar at both temperatures (data not shown). No decrease of DNA fluorescence was observed during the remaining time of the experiment (data not shown).

DISCUSSION

The aim of this study was to investigate changes in cellular states within *Deleya aquamarina* populations submitted to survival-starvation conditions at 5°C and 20°C.

The large decrease in FALS signals at the onset of starvation at 20°C was related to a reduction of the cellular volume, since cells became spherical as observed by microscopy. Such reduction of the cellular volume may result from the degradation of endogenous material and the reorganization of the cellular structure, including changes in membrane composition (33, 35, 40). This size reduction cannot be explained by reductive division (35) due to maintenance in the genomic contents of individual cells. In contrast to cells starved at 20°C, cells starved at 5°C maintained the initial FALS value.

Different physiological states can be defined according to the methods used for their detection (Table 2). The definition of different cellular categories within natural communities is often limited to active versus inactive states (see, e.g., reference 11) or viable versus nonviable states (see, e.g., reference 25). In this study, the analysis of different physiological characteristics has led to the definition of more categories which allow the discrimination of five physiological states. The effect of temperature on the culturability of nonpsychrophilic marine bacteria has rarely been addressed. In the case of *D. aquamarina*, we observed a greater maintenance of culturability at the lowest temperature. In contrast, a negative effect of low temperatures on the plateability of four estuarine pathogenic *Vibrio* species was reported (13, 22, 50, 64). In the case of *V. vulnificus*, this negative effect of temperature was not related to the presence of nutrients (42). For most nonmarine bacteria, culturability seems to be better preserved at lower temperatures under starvation conditions (16, 38, 54), but the opposite observations have also been reported (41, 46). These conflicting

FIG. 3. FCM analysis of cytoplasmic membrane permeability (BacLight kit). The x axis shows green fluorescence, and the y axis shows red fluorescence. Results for ethanol-treated cells (control) (A) and cells after 0 days (B), 52 days (C), and 92 days (D) of starvation at 20°C are shown.

reports may be due to variations in water quality between studies (63). In our study, the CFU counts at 20°C displayed the type C survival pattern described by Morita (35) while CFU counts at 5°C are more related to the type B survival pattern. In both cases, the culturable-cell fraction remained stable from the onset of starvation (at 5°C) or after a slight increase followed by a significant decline (at 20°C). The culturable fraction was stabilized at 10 to 20% and 70% of total direct counts at 20 and 5°C, respectively. The existence of a constant culturable cell fraction during survival-starvation after a initial decrease has already been reported for other marine heterotrophic species, including natural isolates (3), psychrophilic bacteria (36), and oligotrophic bacteria (15). Moyer and Morita (36) described this survival pattern as “indicative only of heterotrophic bacteria adapted to survival for extensive periods of time without significant nutrient input and should not be considered exclusive to all bacteria forms.”

Our results show that the culturable-cell fraction is always equal to or slightly exceeds the fractions of DVC⁺, CTC⁺, and ENZ⁺ cells at both temperatures. Thus, the VBNC state may not represent an important part of the life cycle of these cells under these experimental conditions. Discrepancies observed between CTC⁺ and CFU⁺/DVC⁺ fractions during stage 2 of survival at 20°C and after 42 days of survival at 5°C may be explained by the fact that CTC is a real determination of metabolic activity and that DVC as well as CFU correspond to potential activity assessments. In the case of cells starved at 5°C, the CTC⁺ percentages recorded at 5°C were not improved

when CTC incubations were performed at 20°C (Table 1). Thus, the lower fraction of cells showing a real respiratory activity when compared to the culturable fraction should be due to the lack of energy rather than to low temperature. The better maintenance of cellular activities at the lower temperature may be explained by the fact that maintenance energy is decreased, so that the rate of physiological activity and energy loss are lowered, resulting in a prolonged time of survival.

The absence of VBNC cells was also reported by Amy et al. (4) during the starvation survival of the marine psychrophilic bacterium *Vibrio* sp. strain ANT-300. In contrast, other marine bacteria, such as *Pseudomonas* spp., are able to develop a VBNC state (26). In the case of four estuarine *Vibrio* species (*V. cholerae*, *V. vulnificus*, *V. harveyi*, and *V. parahaemolyticus*), the temperature was considered an inducer of the VBNC state due to the existence of nonculturable cells showing respiratory activity (39, 42), substrate responsiveness (7, 22, 42, 50), or enzymatic activity (17). When such cells are exposed to an upshift of temperature, a large increase of culturable-cell counts was observed without significant modification of total counts. This recovery of culturable cells was initially interpreted as a bacterial resurrection (i.e., reversibility of the nonculturable state) (39) but was later shown to be more probably due to the growth of a few remaining culturable and/or resuscitatable cells (7, 17, 22, 50, 61). Thus, there is no strong evidence that the VBNC state constitutes a favorable strategy for bacteria to survive in cold environments.

The difference between counts of cells showing a metabolic

FIG. 4. Changes in physiological characteristics occurring during survival of *D. aquamarina* at 20°C in two separate experiments (experiments 1 and 2). The characteristics were as follows: (A) total direct counts and culturable-cell counts; (B) percentages of CTC⁺ cells (●), ENZ⁺ cells (○), MEMB⁺ cells (▲), and DVC⁺ cells (△); (C) FALS and DNA content. (All values are relative to that recorded at $T_0 = 1.0$.)

activity (CFU⁺, CTC⁺, DVC⁺, and ENZ⁺) and those with an intact membrane permeability (MEMB⁺) suggests the existence of a subpopulation of nonactive cells with selective cytoplasmic membrane permeability. Such intact (Table 2) but nonactive cells were observed in our study from days 13 to 72 at 20°C. The fraction of intact cells was maximal at day 35, representing 60 to 65% of the population, and then declined to 0% by day 72. The maintenance of membrane permeability may represent the last stage of viability to be detected, occurring with a delay after the loss of other physiological functions, even if the significance of this cellular state remains unclear in terms of viability and recovery. Recently, the absence of intact bacterial cells has been reported in coastal waters by using CTC (active cells) and BacLight (cells with membrane integ-

riety) procedures (9). This suggests that intact bacterial cells, a state which precedes the damaged and dead states, may represent a transition step of low significance within natural communities.

The alteration of membrane permeability leads to the formation of damaged cells (Table 2) with an entire genomic content. These cells represented a large fraction of the population after 72 days of starvation at 20°C. The loss of membrane permeability may expose the cellular components to the extracellular environment, leading inevitably to their degradation (37). This cellular state may represent an irreversible loss of viability which is not related to cell membrane disruption (autolysis) but is most probably related to cytoplasmic lysis and nucleic acid degradation. In contrast, Votyakova et al. (59)

FIG. 5. FCM histograms of the DNA content (HO342 staining) of *D. aquamarina* cells after 0 days (A), 13 days (B) and 92 days (C and D) of starvation at 20°C in two separate experiments. The x axis shows the DNA content (HO342 staining), and the y axis shows the relative cell numbers. Numbers preceding N indicate the genomic content.

showed that some damaged starved cells could restore their membrane permeability after nutrient addition but only a fraction of these cells were able to recover a culturable state. These results suggest that repair and possession of intact membrane permeability are necessary but not sufficient conditions to recover a culturable state. Further experiments on the significance of this labeling technique are needed to clearly understand the reversibility of this cellular state and its significance in terms of viability.

Characterization of dead cells (i.e., cells showing a loss of genomic integrity) (Table 2) should be considered with caution since modification of the fluorescence of the DNA-HO342 complex can result from degradation or topological changes of DNA (28). Moreover, the upper limit used to define this type of cell is not obvious. For instance, when this limit is based on the lower fluorescence value corresponding to one genome content, the number of dead cells may be underestimated in the case of multigenomic cells showing an altered DNA, which remains greater than that of cells containing a single genome. A notable DNA fluorescence alteration was observed only in experiment 2 at 20°C. The increase of the cellular fraction showing altered DNA during stage 3 may result from the gradual increase of the damaged-cell fraction. However, the significance of the damaged-cell fraction is doubtful since no major DNA alteration was observed during experiment 1 at 20°C.

Siegele and Kolter (53) suggested that death may be seen as

a stochastic process in which survivors are a random subpopulation of the initial population or as a deterministic process concerning a fraction of the population which, at the onset of starvation, are at a particular point of their cell cycle that prevents the expression of starvation response mechanisms. Recently, the hypothesis of programmed cell death in bacterial populations (65) and the significance of an excreted factor(s) (61) was put forward. However, even if cell death or survival follows a deterministic model, this does not preclude probabilistic components, leading to some variability in the final results as exemplified by the differences observed between experiments 1 and 2 at 20°C.

Some ecological implications, from both methodological and conceptual viewpoints, can be deduced from the evolution of physiological parameters during starvation survival of *D. aquamarina*. In this study, we showed that starved bacteria sometimes need a prolonged incubation (until 24 h) before demonstrating detectable elongation by the DVC method. In natural waters, the DVC method is based on an incubation no longer than 6 h, due to the division of some nalidixic acid-resistant species (25). Consequently, this method may underestimate the real proportion of viable cells in seawater due to the presence of a large fraction of starved bacteria. It has also been proposed to consider the active fraction of respiring bacteria to be the one responsible for the production of new cells, leading to a more realistic view of how systems function (11, 14, 32). However, different experimental results (4, 9, 26; also see

TABLE 1. Comparative results between physiological characteristics during survival at 20 and 5°C

Physiological characteristic	Mean value (% of total count) $\pm$ SD after ^a :					
	23 days		42 days		92 days	
	20°C	5°C	20°C	5°C	20°C	5°C
CFU	58.4 $\pm$ 0.5	73.3 $\pm$ 5.8	12.6 $\pm$ 6.9	78.6 $\pm$ 4.6	12.7 $\pm$ 8.3	86.3 $\pm$ 14.2
CTC ⁺ (20°C) ^b	30.7 $\pm$ 1.4	89.7 $\pm$ 0.8	15.3 $\pm$ 7.4	68.7 $\pm$ 12.4	21.2 $\pm$ 17.6	40.6 $\pm$ 3.2
CTC ⁺ (5°C) ^c	ND ^d	88.3 $\pm$ 2.0	ND	65.7 $\pm$ 2.1	ND	45.5 $\pm$ 9.6
DVC ⁺	61.4 $\pm$ 4.3	84.7 $\pm$ 3.5	20.0 $\pm$ 12.2	84.3 $\pm$ 0.4	24.8 $\pm$ 14.9	51.1 $\pm$ 0.7
MEMB ⁺	93.4 $\pm$ 0.7	96.8 $\pm$ 0.8	83.0 $\pm$ 3.7	91.9 $\pm$ 0.1	26.5 $\pm$ 12.6	85.4 $\pm$ 2.7

^a Mean $\pm$ SD for duplicate microcosms.

^b CTC incubation performed at 5°C.

^c CTC incubation performed at 20°C.

^d ND, not determined.

TABLE 2. Terms used to describe the five major physiological states defined herein

Term	Physiological state
Culturable cells	Cells that can grow on traditional culture media
Viable but nonculturable (VBNC) cells	Nonculturable cells demonstrating a real or potential metabolic activity
Intact cells	Cells with selective cytoplasmic membrane permeability but without any signs of metabolic activity
Damaged cells	Cells with genetic integrity but without an intact cell membrane
Dead cells	Cells with damaged genetic integrity

above) suggest that respiration should be used with caution since starved nongrowing marine bacteria remain capable of respiration. Moreover, the application of CTC to natural communities may be biased by lack of universality (62) and toxicity (58) for bacteria. Such a limitation can also be applied to the use of BacLight (27).

There is little information on the physiological heterogeneity of starved marine bacteria, and most research has focused on *Vibrio* species, which are considered a major taxonomic group of marine bacterial communities (5). However, the recent development of phylogenetic studies has revealed the existence of a large diversity of organisms (2, 19, 60). The first question is, to what extent is the absence of VBNC forms, reported here for *D. aquamarina*, widespread in marine ecosystems? On the other hand, we do not know whether the existence of the VBNC state is controlled mainly by environmental factors or is only species dependent. Some reports aimed at the detection of one specific organism (immunofluorescence) or at the analysis of phylogenetic diversity of natural communities (DNA- and RNA-based approaches), seem to indicate that some typically culturable bacteria are present in natural communities but are not recoverable by culture techniques (10, 18, 21, 30, 49, 51). However, immunostaining does not relate to the viability of target organisms, and rDNA can be detected in nonviable forms of organisms, as shown in this study by the existence of damaged cells and as shown by Josephson et al. (23). Rehnstam et al. (51) have proposed that the low plating efficiency of culturable bacteria may be due to the inability of phage-infected bacteria to produce a colony on a culture medium where bacteriophages could enter the lytic phase. Thus, viral infection may result in a VBNC-like state which differs from the VBNC state commonly described as a result of environmental stresses.

One of the fundamental challenges for the future is to identify and measure the activities of individual microbial cells within marine natural communities. This effort could contribute to the development of new models of microbial ecology in which bacterial cells are present in different physiological states (8), thus leading to a better understanding of nutrient recycling.

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