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In Situ Analysis of Nucleic Acids in Cold-Induced Nonculturable *Vibrio vulnificus*

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Low-temperature-induced nonculturable cells of the human pathogenic bacterium *Vibrio vulnificus* retained significant amounts of nucleic acids for more than 5 months. Upon permeabilization of fixed cells, however, an increasing number of cold-incubated cells released the nucleic acids. This indicates substantial degradation of DNA and RNA in nonculturable cells prior to fixation. Treatment of permeabilized cells with DNase and RNase allowed differential staining of DNA and RNA with the nucleic acid dye 4′,6-diamidino-2-phenylindole (DAPI). Epifluorescence microscopy revealed that the cold-induced nonculturable populations of *V. vulnificus* are highly heterogeneous with regard to their nucleic acid content. The fraction of nonculturable cells which maintained DNA and RNA structures decreased gradually during cold incubation. After 5 months at 5°C, less than 0.05% of the cells could be observed to retain DNA and RNA. In parallel with the loss of nucleic acids, an increase in the concentrations of UV-absorbing material in the culture supernatants was observed in nonculturable-cell suspensions. It is hypothesized that there are two phases of the formation of nonculturable cells of *V. vulnificus*: the first involves a loss of culturability with maintenance of cellular integrity and intact RNA and DNA (and thus possibly viability), and the second is typified by a gradual degradation of nucleic acids, the products of which partly remain inside the cells and partly diffuse into the extracellular space. A small number of nonculturable cells, however, retain DNA and RNA, and thus may be viable despite having reduced culturability.

The human pathogen *Vibrio vulnificus* is a natural inhabitant of estuarine environments which has been shown to enter the viable but nonculturable (VBNC) state during low-temperature incubation (7, 20–22). Approximately 1 to 10% of cold-induced nonculturable populations of *V. vulnificus* display respiratory activity as assayed by the reduction of tetrazolium salts [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and cyanoditolyl tetrazolium chloride (CTC)] (12, 26, 33) and retain the capacity for elongation in response to substrate addition as assayed by incubation in the presence of nalidixic acid (16, 17). The lack of respiratory activity and the inability to elongate as observed for the majority of the cells in nonculturable populations may be interpreted as either a loss of viability or entry into a state of dormancy (15, 28).

Cellular integrity and the presence of nucleic acids, ribosomes, and machinery for protein synthesis may be considered necessary but not sufficient for viability. The detection of DNA by PCR or hybridization, the detection of rRNA by hybridization (2), and the detection of protein synthesis by means of inducible enzyme activity (19) are techniques which identify cells as potentially viable. Based on these methods, it is apparent that VBNC cells maintain certain characteristics of viable cells, such as the potential for metabolic activity and respiration as well as cellular integrity. However, the detection limits of these techniques do not permit a reliable determination of viability in natural starved or stressed populations.

An alternative method for assessing viability is the determination of the presence of nucleic acids. We have developed a simple method to detect DNA and RNA by differential in situ digestion of DNA and RNA and tested the usefulness of this method by using VBNC suspensions of *V. vulnificus*. In addition, the release of cellular material such as nucleic acids was studied in suspensions of nonculturable cells by assessing the UV absorbance of samples from supernatants of these suspensions. Based on results obtained by this method, we propose the existence of two phases of the VBNC state, the first occurring immediately after a loss of culturability and characterized by maintenance of cellular integrity and the second occurring as a gradual degradation of nucleic acids with an eventual loss of viability in the majority of the nonculturable population. The significance of the results is discussed in terms of the survival of the organism during cold incubation, as well as with regard to the general usefulness of the approach for the study of natural populations.

MATERIALS AND METHODS

Bacterium, incubation conditions, and supernatants. All experiments in this study were performed with *V. vulnificus* C7184T (27). Cells of *V. vulnificus* C7184T were grown in Väätänen nine salts solution (VNSS/2) (23) at 24°C with shaking and harvested in the logarithmic phase of growth by centrifugation at 12,000 × *g* for 15 min at 15°C. The cells were then washed and resuspended in 2M-carbon-nitrogen-phosphorus medium (2M-CNP), a morpholinepropanesulfonic acid (MOPS)-buffered medium which contains the same salts as VNSS/2 but lacks carbon, nitrogen, and phosphorus sources (23). The cell density after resuspension was between 8×10^7 and 4×10^8 CFU/ml. The experiments were performed statically at 24°C for starvation and at 5°C for cold incubation. To obtain supernatants, the cultures were centrifuged at 8,000 × *g* for 30 min at 5°C.

Fixation of bacterial cells. Paraformaldehyde fixative (4% [wt/vol] [1]) was prepared as follows. Twenty microliters of 10 M NaOH was added to 16.5 ml of water which had been heated to 60°C in a 75-ml flask, followed by 1 g of paraformaldehyde. After dissolution of the paraformaldehyde, 8.3 ml of 3× phosphate-buffered saline (PBS) (390 mM NaCl, 30 mM $NaPO_4$ buffer [pH 7.2]) was added. The solution was cooled to 0°C, adjusted to a pH of 7.2, filter sterilized, and used within 24 h.

One-milliliter aliquots of cell culture were harvested by centrifugation at 8,000 × *g* at 5°C for 30 min, after which the cell pellets were resuspended in 250 μl of the supernatant. Fresh paraformaldehyde fixative (750 μl) was added, and the tubes were vortexed for 1 min and incubated at 5°C for 20 h. The cells were pelleted at 8,000 × *g* and 5°C for 30 min, the supernatant was decanted, and the pellet was resuspended in 450 μl of 1× PBS. Fifty microliters of 0.1% Nonidet

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P-40 (NP-40) was added, and the cells were vortexed, pelleted, resuspended in 0.1% NP-40, pelleted again, and resuspended in 30 μl of 2× storage buffer (40 mM Tris, 0.2% NP-40 [pH 7.5]). An equal volume of 100% ethanol was added, and the cells were stored at −20°C.

Hybridization. Cells were fixed with paraformaldehyde as described above and hybridized with a fluorescein isothiocyanate-labeled eubacterial probe, EUB338, directed against the 16S rRNA (10), by standard procedures (1) with the following parameters: a probe concentration of 2.5 μg/μl, 30% formamide, and incubation at 37°C for 2 h.

Permeabilization of cells. One to two microliters of the fixed cell suspensions was spread on clean six-well Teflon-coated glass hybridization slides (Cel-Line Associates Inc., Newfield, N.J.) and allowed to air dry for 1 h. The slides were placed for 2 min in a 50, 90, and 100% ethanol wash series and dried. Slides were cooled to 0°C, and 40 μl of ice-cold lysozyme solution (5 mg of lysozyme [Boehringer, Mannheim, Germany] ml^{-1} in 100 mM Tris-HCl, pH 7.5) was added to the cell smear. After 10 min of digestion, the slides were washed for 2 min in 0°C TE (10 mM Tris-HCl, 0.1 mM EDTA [pH 7.5]) and for 2 min in a 50, 90, and 100% ethanol series and allowed to air dry.

DNA and RNA digestion. Twenty-five microliters of the DNase mixture {2.5 μl of 10× buffer (100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol [pH 7.5]), 2.5 μl of 100 mM $CaCl_2$, 2.5 μl of 10-mg ml^{-1} bovine serum albumin, 5 U of RQ1 RNase-free DNase (Promega, Madison, Wis.)} and H_2O to 25 μl were added to the permeabilized fixed cells. Individual slides were carefully placed horizontally into a 50-ml Falcon tube containing a small amount of damp tissue paper and incubated at 37°C for 1 h. The RNase digestion was performed as described above, with 25 μl of RNase mixture (50 mM Tris-HCl, 10 mM EDTA, 100 μg of RNase A [Promega] ml^{-1} [pH 7.5]) added to the cells for 30 min at 25°C. The slides were washed for 2 min in 50, 90, and 100% ethanol before staining was performed with 10 μg of 4′,6-diamidino-2-phenylindole (DAPI) ml^{-1} in distilled water for 2 min, followed by washing with 100% ethanol.

Staining with acridine orange, ethidium bromide, and Hoechst 33258. Samples were fixed with formaldehyde (2% [wt/vol]) and stained with 0.01% (wt/vol) acridine orange, 50 μg of ethidium bromide ml^{-1} (in the presence of 0.1% Triton X-100 [31]), or 10^{-5} M Hoechst 33258 (24) for 20 min in the dark. At least two subsamples each were filtered onto prestained black polycarbonate filters (Meclos Company), washed with filtered (0.2-μm pore size) distilled water, or, in the case of nonfixed cells, with filtered 2M medium. If no staining could be observed, the duration of staining was extended to 3 days at 5°C. Negative results were confirmed with at least three independent samples. For permeabilization, cells were pelleted, resuspended in 0.5 mM $MgSO_4$, and electroporated in the presence of the nucleic acid stain (*E. coli* Pulser, 0.2-cm-diameter cuvettes; Bio-Rad). In order to visualize lysis products, 2% formaldehyde was added immediately following resuspension in distilled water or sonication for 90 s, followed by the addition of 10^{-5} M Hoechst 33258 for 2 min and filtration onto prestained black filters. The specificity of the stain was tested by addition of 0.1 or 1 mg of DNase ml^{-1} for 20 or 40 min prior to fixation.

Visualization. Microscopy was performed with an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) fitted with an HBO 50-W mercury lamp and a 100× 1.3-numerical-aperture oil immersion lens. Cells were visualized under UV illumination with filter set 10 (Carl Zeiss), and the fraction of stained cells was determined by counting at least 200 cells (in the case of low percentages of stained cells, 2,000 cells) per sample. Photographs were taken with Ektachrome 400 film (Eastman Kodak, Rochester, N.Y.).

Determination of CFU. To assess the culturability of C7184T, samples were taken at the times indicated below and diluted in 2M-CNP. Drop plate counting (13) was performed on agar plates prepared with VNSS/2. Plates were incubated for 24 h at 24°C before the determination of CFU. After incubation for a total of 7 days, no further colony development could be observed.

Extraction of DNA. DNA extractions were performed as described by Chomczynski and Sacchi (5). Briefly, cells were pelleted and the pellet was resuspended in 1 ml of guanidinium thiocyanate which had been preheated to 65°C. After lysis of cells, 1 ml of buffered phenol preheated to 65°C was added to the suspension, followed by 2.5 ml of chloroform. The solution was mixed vigorously several times while incubated at 65°C. The solution was then placed on ice for 10 min, the aqueous layer was removed, and the DNA was precipitated with ethanol. DNA was analyzed on a 1% agarose gel.

RESULTS

Staining of *V. vulnificus* cells in different growth stages. The results of staining of cells of *V. vulnificus* by different methods are summarized in Table 1. Cells in all physiological stages tested displayed staining with all of the stains, but the protocols required for staining depended on the age of the cells. After fixation with formaldehyde, young VBNC cells (cells kept at 5°C for 25 days) were readily stained with all stains employed. Old VBNC cells (cells kept at low temperature for 150 days), on the other hand, had to be permeabilized by means of electroporation in order to achieve staining of the cells with Hoechst 33258. Lysis of these cells by means of sonication or osmotic shock released material that was stained by Hoechst 33258 and was sensitive to DNase.

TABLE 1. Staining of cells of *V. vulnificus* C7184T in different states with or without prior fixation with formaldehyde

Stain	Permeabilization and fixation with 2% formaldehyde	Exponentially growing cells	Young VBNC cells (25 days)	Old VBNC cells (150 days)
DAPI	+	Yes	Yes	Yes
	−	No	No	No
Acridine orange	+	Yes	Yes	Yes
	−	Yes	Yes	No
Ethidium bromide	+	ND[a]	Yes	Yes
	−	ND	Yes	No
Hoechst 33258	+	Yes	Yes	No
	−	No	No	Yes[b]

[a] ND, not determined.
[b] Staining possible after electroporation.

Hybridization of cells with a 16S rRNA-specific probe. Cell samples which hybridized with fluorescently labeled eubacterial 16S rRNA probe EUB338 (10) showed that while growing cells gave strong signals, the signal intensity decreased with incubation time at 5°C: after 12 days, only 10 to 20% of the cells gave strong signals, and more than 50% of cells were not detected (data not shown); after 26 days at 5°C, less than 0.1% of cells could be detected by hybridization. After 120 days at 5°C, no signal could be detected by microscopy of hybridized samples in any of the cells.

Staining with DAPI after differential digestion with DNase and RNase. Paraformaldehyde-fixed and permeabilized cells were subjected to DAPI staining after in situ digestion of DNA or RNA. After DNase treatment, exponentially growing cells were clearly stained (Fig. 1b), reflecting the RNA content of the cells. Samples that had been digested with both DNase and RNase showed very little staining, indicating that the permeabilization and digestion were efficient and that staining with DAPI was specific for nucleic acids (Fig. 1c).

Cells that had been kept at 5°C for 15 to 33 days, at which time the CFU counts were less than 10^{-5}% of the initial population, showed considerable heterogeneity concerning the presence of nucleic acids in fixed and permeabilized cells. After 33 days, about 50% of the population were stained to various degrees but the remaining 50% of the cells displayed no staining (Fig. 1d). After treatment with DNase, about 20% of the population were still stained by DAPI (Fig. 1e).

All cells in nonculturable populations kept at 5°C for 160 days (old VBNC cells) were stained by DAPI after fixation with paraformaldehyde and treatment with NP-40 as described in Materials and Methods (data not shown); after permeabilization with lysozyme, however, only a small fraction (about 0.5%) of cells were stained (Fig. 1f). After DNase treatment of permeabilized cells, less than 0.05% of old VBNC cells retained DAPI-stainable material (Fig. 1g), and after DNase and RNase treatment, no staining was observed (data not shown). These results indicate that nucleic acids, in particular RNA, are degraded prior to fixation in the majority of old VBNC cells and that only a very small proportion of the populations maintain DNA and RNA during long-term cold incubation.

Release of nucleic acids during cold incubation. The supernatants of cold-incubated cells were examined by UV spectroscopy at 260 and 280 nm and were found to contain between 10.9 and 41 μg of nucleic acid material per ml. During cold incubation, the concentration of nucleic acids in the superna-

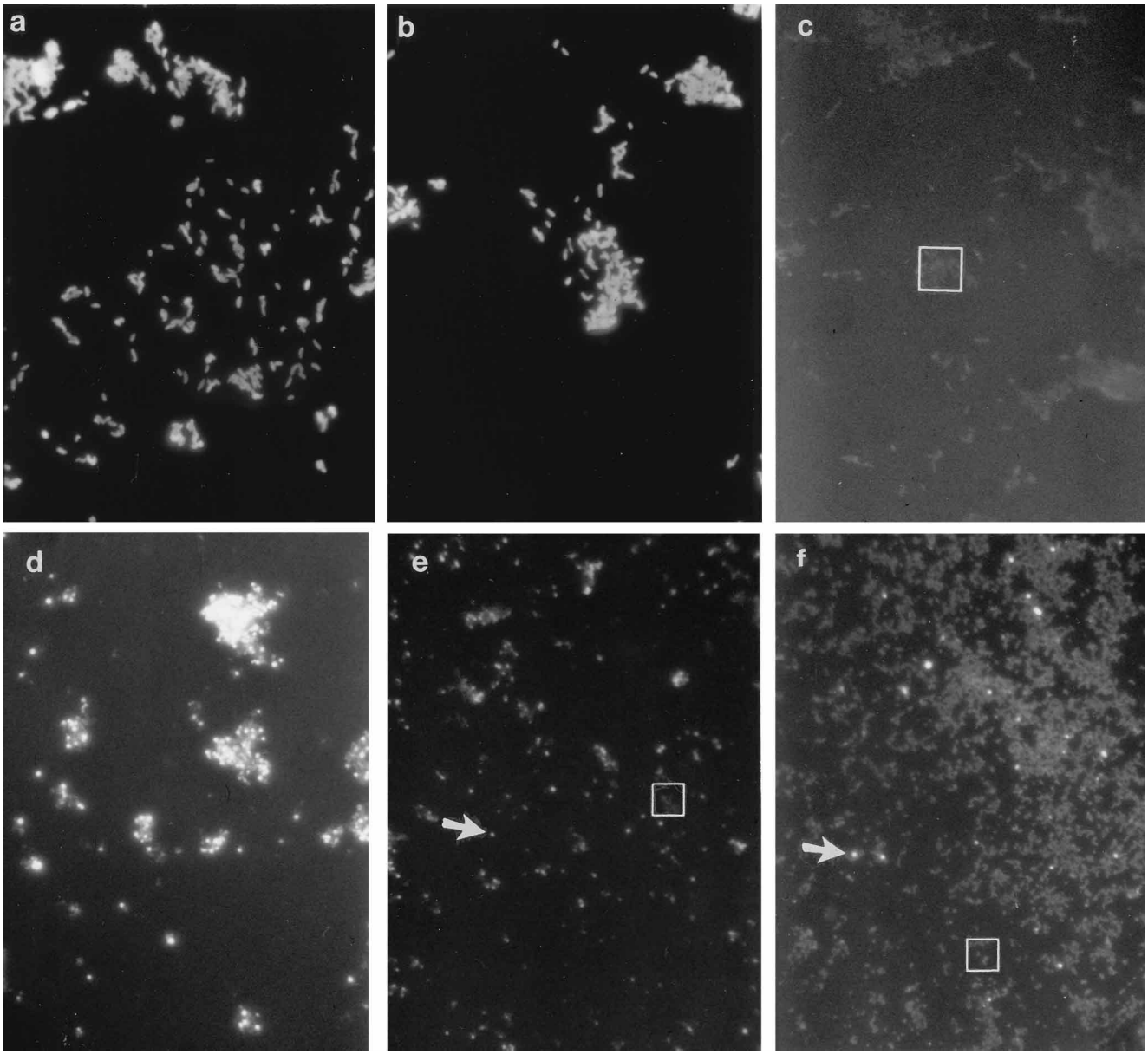

FIG. 1. Epifluorescence microscopic images of DAPI-stained cells of *V. vulnificus* C7184T during exponential growth (a to c), after 33 days at 5°C (d and e), and after 160 days at 5°C (f and g). The cells were subjected to fixation with paraformaldehyde and permeabilization with lysozyme as described in Materials and Methods and were subsequently treated with DNase (b, e, and g) or with DNase and RNase (c) or left untreated (a, d, and f) prior to staining with DAPI. Representative fluorescent (arrow) and nonfluorescent (box) cells are indicated.

tants increased with time until about 50 days, after which there was little increase.

Degradation of DNA during cold incubation. DNA extracted from samples which had been cold incubated for 25 to 30 days was compared to DNA extracted from logarithmic-phase cells. Logarithmic-phase cells maintained intact chromosomal DNA, while the VBNC cells contained degraded DNA, as shown in Fig. 2, suggesting the loss of viability of a significant portion of the population.

DISCUSSION

In this study, we have assessed the viability of nonculturable cells of *V. vulnificus* using the presence of intact DNA and RNA as indicators of potential viability. *V. vulnificus* serves as a useful model for study because it displays maintenance of culturability during starvation at moderate temperature, exhibiting a typical starvation-stress response, and also displays a loss of culturability (VBNC response) following a shift to low temperature, thus allowing the study of both responses in the same organism.

Specific in situ staining, or the lack thereof, before or after DNase or RNase treatment demonstrates that DAPI is a reliable stain for nucleic acids. Cold-induced VBNC cells of *V. vulnificus* retain cellular stability and significant amounts of DAPI-stainable material even after prolonged cold exposure (30). Further confirmation that VBNC cells which have been cold incubated for 150 days contain DNA has been obtained by utilization of stains such as acridine orange, ethidium bromide, and Hoechst 33258.

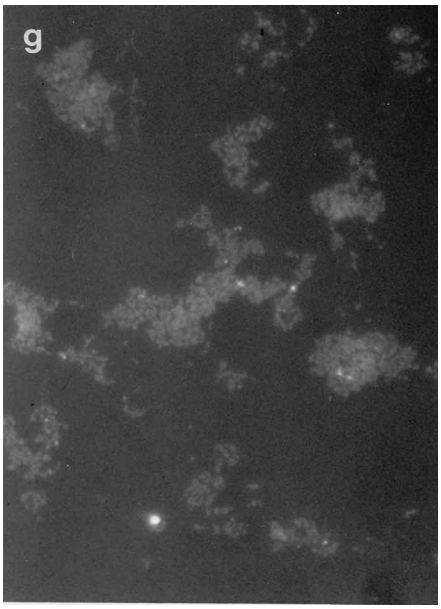

FIG. 1—*Continued.*

Several days after the loss of more than 99.999% of culturability, a significant fraction of the nonculturable population displayed maintenance of DNA and RNA, as detected by DAPI staining and hybridization with a 16S rRNA probe. Both methods indicated considerable heterogeneity among these young VBNC cell populations regarding their nucleic acid content. DAPI staining was observed to be more sensitive than hybridization: less than 0.1% of the cells could be detected by hybridization after 26 days at 5°C, while 20% of the cells contained RNA detectable by DAPI staining after 33 days of cold incubation (Fig. 1e). DAPI readily penetrates fixed cells even without prior permeabilization with lysozyme, while hybridization with 16S rRNA probes may be limited by the permeability of cells due to the sizes of the probes employed.

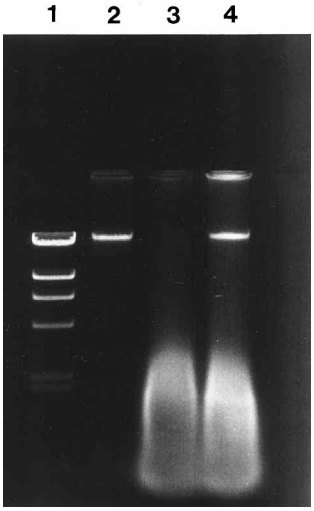

FIG. 2. Electrophoretic image of chromosomal DNA extracted from logarithmic-phase and nonculturable cells of *V. vulnificus*. Lane 1, molecular weight marker; lane 2, logarithmic-phase cells; lane 3, 30-day-old nonculturable cells; lane 4, 30-day-old nonculturable cells spiked with logarithmic-phase cells prior to extraction.

Permeabilization of paraformaldehyde-fixed nonculturable cells which had been incubated for a long period induced the rapid release of DAPI-stained nucleic acids by 99.5% of the population, indicating degradation of DNA and RNA in these cells. The fraction of the population with detectable DNA and RNA after DAPI staining of fixed and permeabilized cells decreased with incubation time at 5°C; after 150 days at 5°C, less than 0.05% of the cells gave a signal. Further evidence for the loss of intact nucleic acids was obtained by the extraction and visualization of nucleic acids by gel electrophoresis. These results indicate progressive, significant degradation of the chromosome and ribosomes in aged cold-incubated cells.

During cold incubation, nonculturable cells were found to release significant amounts of UV-absorbing material, which possibly originated from degradation of ribosomes and chromosomes. The delay in the loss of culturability of cells that had been starved prior to cold incubation is reflected in a delayed accumulation of UV-absorbing material in the supernatants of these cells. Thus, the loss of ribosome and chromosome integrity may be correlated to the release of UV-absorbing material in nonculturable cells of *V. vulnificus*. Therefore, the loss of culturability may be a consequence of the degradation of nucleic acid material.

It has been reported that the PCR amplification of species-specific DNA sequences in samples of VBNC cells of *V. vulnificus* requires over 10,000 times more extracted DNA than the amplification of the same sequence in samples of growing cells (3). It appears that the degradation of DNA in aged VBNC cells is responsible for the lack of amplification of specific DNA sequences.

Degradation of RNA and release of the degradation products have been reported to occur in populations of starved or stressed bacteria (4, 6, 8, 18, 25, 29). In contrast, *Vibrio* sp. strain S14 and *Pseudomonas putida* maintain ribosomes during carbon starvation (9, 11). Furthermore, it is interesting that while survival (culturability) of *Vibrio* sp. strain S14 and *P. putida* during carbon starvation is not impaired (11, 14), the loss of ribosomes in these two strains coincides with a moderate or dramatic loss of viability.

In this study we have shown that DAPI staining of bacterial cells is a sensitive and reliable method for detecting RNA and DNA. Only 0.1% of VBNC cells could be detected by hybridization methods, while more than 20% of cells could be shown to contain detectable amounts of RNA by DAPI staining. It was recently suggested that the staining of formaldehyde-fixed samples of high salinity with DAPI (34) results in the artifactual detection of non-nucleoid-containing ghosts. In the present study, we have attempted staining of nucleoid-containing cells (34) as described previously and conclude that cells of *V. vulnificus* which contain DNA (including cells which are culturable) cannot be detected by the staining protocol used for nucleoid-containing cells and that DAPI staining is a sensitive, reliable means for detection of nucleic acids.

Using our technique, we have demonstrated that VBNC cells of *V. vulnificus* contain significant amounts of DAPI-stainable material even after prolonged cold exposure but that cold incubation for a longer duration results in the progressive loss of RNA and DNA. Recently, similar results have been reported for nonculturable populations of *Legionella pneumophila* (32). Nonculturable cells of this organism retain intact RNA and DNA (as visualized by gel electrophoresis) which became degraded during long-term incubation. The use of differential staining of DNA and RNA in this study has allowed us to demonstrate that this is indeed the case with *V. vulnificus*. We conclude that there are two phases of the VBNC state: (i) the loss of culturability with maintenance of cellular integrity

and intact RNA and DNA (and thus possibly viability) and (ii) degradation of RNA and DNA resulting in the loss of viability. Thus, since the majority of long-term-, cold-incubated cells of *V. vulnificus* do not contain detectable amounts of intact ribosomes or chromosomes, they may not be viable. The integrity of ribosomes and nucleic acids, however, may be maintained in a small fraction of the population for much longer than would be anticipated based on the ability of the cells to grow and divide, and these cells may retain the ability to recover and infect a suitable host. Quantitative and qualitative determinations of the nucleic acid content of nonculturable cell populations clearly require further investigation.

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