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Methionine Uptake and Cytopathogenicity of Viable but Nonculturable *Shigella dysenteriae* Type 1

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A pathogenic strain of *Shigella dysenteriae* type 1 was selected for study to elucidate the physiology and potential pathogenicity of organisms in the viable but nonculturable (VBNC) state in the environment. Studies in our laboratory have shown that *S. dysenteriae* type 1 survives in laboratory microcosms in the VBNC state for long periods of time, i.e., more than 6 months. VBNC cells of *S. dysenteriae* type 1 were found to retain cytopathogenicity for cultured HeLa cells. To determine whether VBNC *S. dysenteriae* type 1 expressed protein after loss of culturability, ^{35}S-labelled methionine was added to suspensions of VBNC cells. Total cellular proteins were extracted and examined by autoradiography. Results indicate that VBNC *S. dysenteriae* type 1 is capable of both active uptake of methionine and incorporation of methionine into protein. Amino acid uptake and protein synthesis substantiate the viability of cells of *S. dysenteriae* type 1 in the VBNC state, i.e., although the cells are unable to be cultured on laboratory media by standard bacteriological methods, the cells remain metabolically active. Furthermore, VBNC cells of *S. dysenteriae* type 1 may pose a potential public health hazard that has not yet been recognized.

The viable but nonculturable (VBNC) state of nonsporulating gram-negative bacteria is, as yet, poorly understood. Believed to be a complex physiological adaptation strategy, the VBNC condition allows bacteria to remain viable for extended periods of time, essentially in a survival stage, during suboptimal growth conditions. Organisms in this state respire actively and maintain a low level of metabolic activity but, in general, do not divide on conventional bacteriological media. Nonculturable microorganisms are assessed for viability by the direct viable count (DVC) method and/or DVC with a tetrazolium salt (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride) (INT). When conditions become favorable, these organisms regain their ability to grow and form colonies on a suitable medium. Many bacterial species, including *Escherichia coli*, *Vibrio cholerae*, *Salmonella enteritidis*, *Aeromonas salmonicida*, *Shigella sonnei*, *Shigella flexneri* (4), *Legionella* spp. (9), *Campylobacter jejuni* (18), and *Helicobacter pylori* (20), have been shown to undergo transition to the VBNC state. A variety of specific growth regimens have been utilized to induce each of these microorganisms to enter the VBNC state. Changes during or after transition to the VBNC state include changes in the size and shape of the bacteria (13, 19), number of ribosomes, short-chain fatty acid content of the membrane (13), virulence pattern (5, 9, 13), etc. Pathogens in the VBNC state are a potential unrecognized reservoir of disease and may constitute a public health hazard. Although it is premature to cite public health implications, several lines of evidence indicate conceivable importance. For example, (i) experiments with animal models have shown that VBNC *V. cholerae* and enteropathogenic *E. coli* are able to regain culturability after animal passage (4); (ii) human volunteers develop clinical symptoms of cholera after ingestion of VBNC *V. cholerae*, with the pathogen subsequently being isolated in the culturable state from stools of the volunteers (5); and (iii) VBNC *Legionella pneumophila* has been demonstrated to resuscitate by growth on chick embryos (9). *Vibrio vulnificus* was claimed to resume culturability upon resuscitation by elevation of temperature (15), but later studies revealed that the work was not reproducible and that temperature-induced reversion was not resuscitation but replication of a few culturable cells remaining in the cell suspensions (24).

A key step in understanding the metabolic and pathogenic potential of cells in this state is to be able to resuscitate VBNC pathogens. Resuscitation or reversibility to the culturable state is not a well-defined event and appears to be unique for each organism. Furthermore, it is complicated by a multitude of factors that include species and strain variation, dormancy stage, nutrient status, salinity of the growth media or environment, temperature, etc. Long-term survival of bacteria under conditions not conducive to active growth, such as those favoring entry to the VBNC state, is important not only from the public health point of view but also for understanding the persistence and epidemiology of nonsporulating pathogens in the environment. As a generalized phenomenon, the VBNC state may prove highly significant for the ecology of gram-negative bacteria.

Shigella dysenteriae type 1 is considered to be the most virulent of the shigellae, since as few as 10 organisms suffice to cause shigellosis, a very severe form of dysentery, in both humans and subhuman primates. Shigellosis can be fatal for malnourished children, and epidemics occur most commonly where hygiene is inadequate and other infections are prevalent (7).

Until recently, there was no evidence of VBNC *S. dysenteriae* type 1 (10). Although other *Shigella* spp. have been studied (4), this study was originally undertaken to develop methods for detection of VBNC shigellae and to develop an in vitro assay for determining virulence of VBNC shigellae. Questions addressed in this study were as follows. (i) Are VBNC cells capable of taking up amino acids? (ii) If they are, do amino acids become incorporated into proteins? (iii) Does *S. dysenteriae* type 1 retain its cytopathogenicity for HeLa cells in the

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VBNC state? We present here a comparative evaluation of the metabolic activity and cytopathogenicity of a virulent strain of *S. dysenteriae* type 1 in the VBNC state versus the culturable state.

MATERIALS AND METHODS

Bacterial strain. The virulent strain of *S. dysenteriae* type 1 (14731) used in this study was isolated from a shigellosis patient at the International Center for Diarrhoeal Disease Research, Bangladesh, Clinical Research Center. The isolate was stored at −70°C in Trypticase soy (TS) broth containing 15% glycerol. For routine culturing, the organism was grown at 37°C on TS agar plates for 18 h.

Inoculation of microcosms. The organism was grown overnight in TS broth, harvested, washed twice in membrane-filtered (0.05-μm-pore-size filter; Poretics Corp., Livermore, Calif.) and autoclaved MilliQ water (Millipore Corp., Bedford, Mass.), and resuspended in 1 liter of the water to a final cell concentration of 10^5 to 10^8 CFU/ml. Microcosms were incubated at selected temperatures, 4, 10, 15, 20, 25, 30, and 35°C, with or without shaking at 100 rpm. All microcosms were examined every third day for culturability by transfer to TS agar plates, which were incubated at 35°C for 5 days, after which the plates were examined for growth. When no culturable cells were obtained on TS agar plates for two consecutive samples, 10- and 100-ml microcosm samples were filtered, after which filters were placed on TS agar plates and incubated for 5 days. Nonculturability was confirmed by transferring the microcosm sample (10 ml) to 1% nutrient broth and TS broth and also by the three-test-tube most-probable-number method (1).

For each sample monitored over time, a slope was computed. This measured the average decline in log units (plate counts) per day. These slopes were analyzed by a two-way analysis of variance (SAS system, release 6.08) with shaking and initial inoculum levels as factors. All tests were performed at an α value of 0.05.

Bacterial counts. Bacteria were enumerated directly by the acridine orange direct count (AODC) method of Hobbie et al. (8).

Viability assays. Viability of the nonculturable cells was tested by the DVC procedure of Kogure et al. (11) and by a modified DVC method using INT, a tetrazolium salt. Yeast extract (YE) (0.02%) and cephalexin (10 μg/ml) were added to nonculturable cells (1 ml), and the mixtures were incubated at 37°C with shaking at 100 rpm for 6 to 8 h. The cells were fixed with formalin (2.0%) and enumerated by the AODC method (8). Viable cells respond to this treatment by becoming elongated in the presence of the YE and antibiotic. The degree of elongation can be observed by comparison with the nontreated acridine orange (AO)-stained cells. For the modified DVC, INT (0.1%) was added, along with YE and the antibiotic. Actively respiring bacteria utilize the salt, converting it to a formazan and thereby producing black pigment within the cells (25). The black deposits are clearly visible within the elongated AO-stained cells under the epifluorescent microscope.

Indirect immunofluorescent-antibody assay. Five microliters of microcosm sample was placed on glass slides and allowed to air dry. The slides were then fixed in methanol. Forty microliters of rhodamine isothiocyanate (Difco) (1:20 dilution in 0.1 M phosphate-buffered saline [PBS]) was added to each well, and the slides were incubated in a dark, moist chamber to block nonspecific binding. The slides were then rinsed in 0.1 M PBS and air dried. Forty microliters (1:100 dilution) of *S. dysenteriae* type 1 polyclonal antiserum (Wellcome Diagnostics) was added, and the mixture was incubated at 37°C for 1 h in a moist, dark chamber. The slides were rinsed, 40 μl of fluorescein isothiocyanate-conjugated goat anti-rabbit serum (1:400 dilution; Sigma, St. Louis, Mo.) was added to the wells, and the slides were incubated for 30 min. The slides were washed, air dried, and mounted under a coverslip with a drop of Bacto FA mounting fluid (pH 9.0) (Difco), after which the slides were observed with an epifluorescent microscope (Carl Zeiss, Inc., Oberkochen, West Germany) (10).

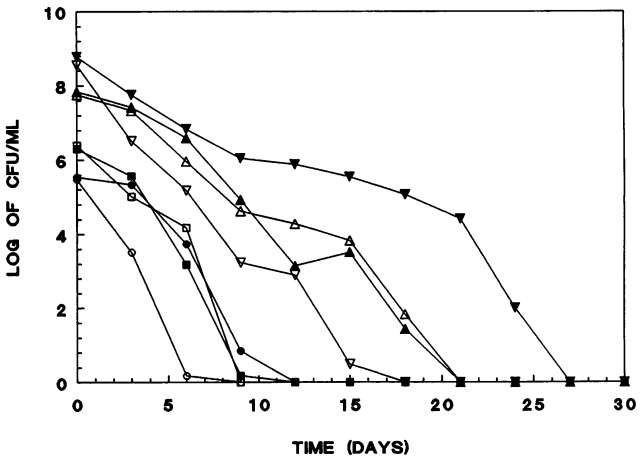
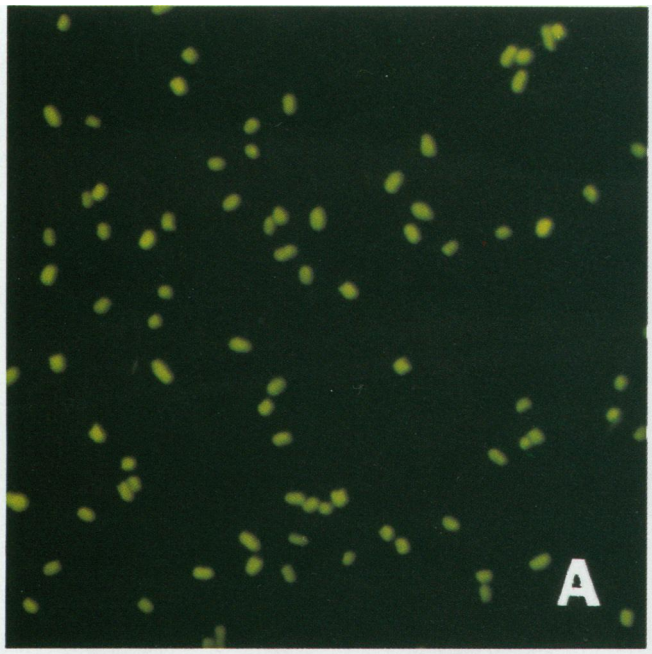
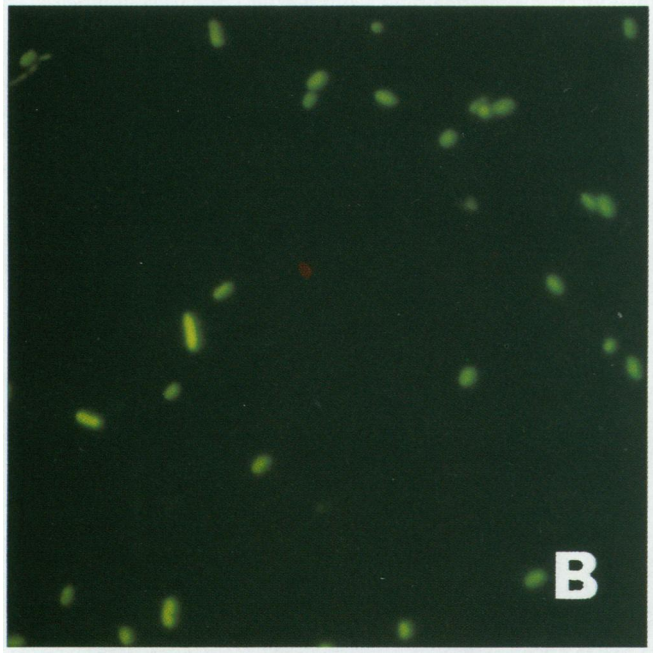


FIG. 1. Effect of cell concentration and aeration on survival of *S. dysenteriae* type 1 by viable plate count assay. Values for initial cell concentrations of 10^5 CFU/ml with (○) and without (●) shaking, 10^6 CFU/ml with (□) and without (■) shaking, 10^7 CFU/ml with (△) and without (▲) shaking, and 10^8 CFU/ml with (▽) and without (▼) shaking are shown. Values were obtained from three independent experiments.

Cytotoxicity assay. The HeLa cell line (ATCC CCL2) was maintained in Eagle's minimal essential medium (EMEM) containing Earle salts and 10% fetal bovine serum (BioWhittaker Products, Walkersville, Md.) at 37°C and 5% CO_2 in a moist environmental chamber. VBNC *S. dysenteriae* type 1 cells (10^7 cells per ml; 4 weeks after entering the VBNC state) were washed twice in PBS and resuspended in the same volume of EMEM. As a positive control, *S. dysenteriae* type 1 was grown in TS broth for 18 h, after which the cells were harvested, washed twice in PBS, and resuspended in EMEM such that the final cell concentration was 10^7 CFU/ml. Boiled cells suspended in EMEM (10^7 cells per ml) were used as a negative control. One hundred microliters of each of these suspensions was used to inoculate monolayers of HeLa cells, and mixtures were incubated in a humidified environment at 37°C in the presence of 5% CO_2. After incubation for 18 h, the HeLa cells were examined microscopically for shrinkage, detachment, and/or cell death.

Labelled amino acid uptake assay. Samples (50 ml) of cells that were VBNC for 4 to 8 weeks, one sample at a concentration of 10^7 cells per ml and a second at 10^6 cells per ml, and a 50-ml sample of culturable cells from freshly inoculated microcosms (10^7 CFU per ml) were used for amino acid uptake experiments. All samples were incubated with 1.0 μCi of ^{35}S-labelled methionine (Amersham Life Sciences, Arlington Heights, Ill.) per ml for 3 days at 37°C. Each sample was run in triplicate, with the duplicate being treated with 2.0% formalin, serving as the negative control, and the triplicate sample being incubated under the same conditions but without any labelled





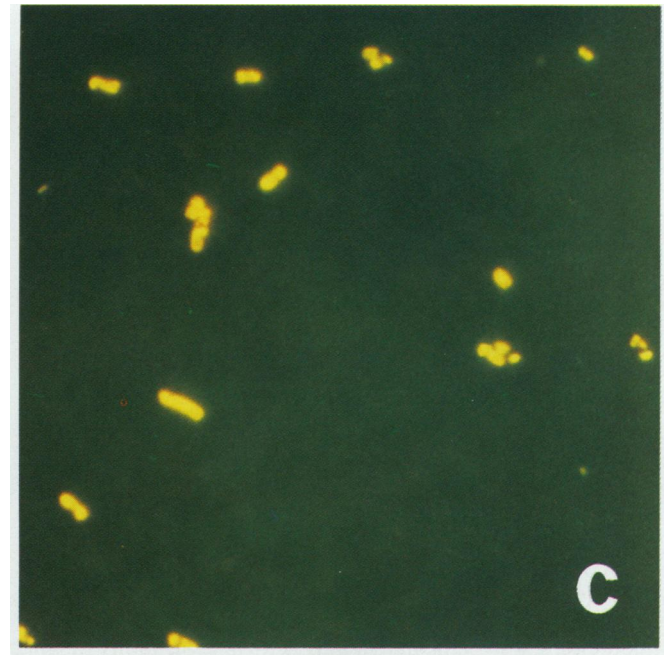


FIG. 2. Photographs of VBNC *S. dysenteriae* type 1 stained with AO. (A) Control VBNC *S. dysenteriae* type 1 (12 weeks after entering the VBNC state). (B) VBNC *S. dysenteriae* type 1 incubated with YE (0.025%) and cephalexin (10 μg/ml) at 35°C for 8 h (DVC). (C) VBNC *S. dysenteriae* type 1 incubated with YE (0.025%), cephalexin (10 μg/ml), and INT (0.1%) at 35°C for 8 h (INT-DVC).

methionine and formalin to confirm nonculturability of the VBNC cells. At 24-h intervals, labelled cells were collected by filtering 1 ml of sample through a 0.2-μm-pore-size Nucleopore membrane, after which radioactivity of the filter was measured. Ten milliliters of the sample from day 2 was centrifuged at 3,000 × *g*, and the pelleted cells were boiled in lysing buffer (50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol; 2% sodium dodecyl sulfate [SDS]; 0.1% bromophenol blue; 10% glycerol) for 10 min. Ten microliters of each sample was used for analysis by SDS-polyacrylamide gel electrophoresis (12) and autoradiography.

A split-plot analysis of variance was used to analyze the data (22). The whole-plot effects were the trial effect and those for the three different microcosms. The trial effect measures the difference in results for the entire experiment repeated at different times. The microcosm effect measures the difference in results for the three treatments: two treatments consisting of two different levels of VBNC *S. dysenteriae* 1 (10^7 and 10^6 cells per ml) and a third treatment with the same pathogen in a culturable state (10^7 cells per ml). The subplot effects were the presence or absence of formalin and uptake of methionine. The same samples were observed daily over a 3-day period. Rather than analyzing data for each day separately, we computed three quantities: a 3-day average, a linear trend, and a quadratic trend. The linear trend is equivalent to a slope describing change over time (6). A significant quadratic trend would be indicative of an initial rise in counts followed by a fall. All data were $\log_{10}$ transformed prior to analysis. All tests were performed at the 5% level of significance.

RESULTS

Cell suspensions of *S. dysenteriae* type 1, at a final concentration of 10^5 CFU/ml, were inoculated into microcosms and incubated at selected temperatures, with shaking at 100 rpm. It was observed that cells incubated at 30 and 37°C became nonculturable by days 12 and 21, respectively. There was little or no change in the plate counts of cells incubated at 4, 10, 15, and 20°C, and these cells remained culturable for the entire period of observation, i.e., 1 month. These observations were also confirmed by the statistical analyses. When samples were enumerated by AODC, however, it was found that all suspen-

TABLE 1. Results of cytotoxicity assay

Sample type	% HeLa cells showing indicated effect[a]		
	A	B	C
S. dysenteriae type 1[b]	++++	++++	++++
S. dysenteriae type 1[b] (freshly inoculated microcosm)	+++	++++	++++
S. dysenteriae type 1[b] (VBNC)	+++	+++	++
S. dysenteriae type 1[b] (heat killed)	−	−	−
EMEM[c]	−	−	−

[a] A, cell detachment; B, cell shrinkage; C, cell death. ++++, 100% of cells showed indicated effect; +++, 75%; ++, 50%; +, 25%; −, 0%.

[b] Cell suspensions were adjusted to a concentration of 10^7 organisms per milliliter. One hundred microliters of each sample was used to inoculate wells in a 96-well microtiter plate.

[c] Uninoculated Eagle's minimal essential medium (control).

sions maintained their initial cell counts. These samples were run in duplicate and observed for a period of 1 month.

On the basis of results from the first set of experiments, 30°C was selected as the optimum temperature for inducing cells of *S. dysenteriae* type 1 to enter the nonculturable state. When microcosms were inoculated with cell concentrations ranging from 10^5 to 10^8 CFU/ml and incubated both with shaking at 100 rpm and without shaking, the following results were obtained (Fig. 1). In microcosms with cells at 10^6 and 10^7 CFU/ml, cells became nonculturable within 9 and 21 days, respectively, regardless of whether shaking was employed. However, in microcosms inoculated with 10^5 and 10^8 CFU/ml and incubated as stationary cultures, cells entered the nonculturable state earlier (9 and 18 days, respectively) than those in shaken cultures (days 12 and 21, respectively). AODC data showed no significant change in cell numbers.

Samples were taken from microcosms at days 5, 10, 15, and 90. At day 5, although the plate counts were lower than plate counts at day 0, about 40% of the cells were still culturable. By day 10, most of the cells lost their culturability. On day 15, no culturable cells were detected by plating on agar media. However, cell morphology of AO-stained *S. dysenteriae* type 1, when examined by epifluorescent microscopy, did not appear to be significantly altered. The size of the cells gradually reduced after incubation for 3 months at 30°C with shaking at 100 rpm (Fig. 2A). To determine viability of the nonculturable cells, DVC and INT-DVC tests were performed for all samples. As expected, viable cells responded by elongating in the presence of YE and the antibiotic (Fig. 2B), and in the modified DVC test, actively respiring cells took up INT and deposited black pigment within the elongated cells (Fig. 2C), a change visible by epifluorescent microscopy. VBNC cells were easily serotyped, by using commercially available rabbit polyclonal sera (Wellcome Diagnostics) and employing the indirect immunofluorescent-antibody method.

VBNC cells of *S. dysenteriae* type 1 (10^7 cells per ml; 4 weeks after entering the VBNC state), when assayed for cytopathic effect on cultured monolayers of HeLa cells (CCL-2), were found to be positive (Table 1), causing cell detachment, shrinkage, and cell death. No such effect was observed with the negative control. Since VBNC cells could not be assayed by viable plate counts, the cells were enumerated by the AODC method.

In the amino acid uptake assay (Fig. 3), VBNC cells of *S. dysenteriae* type 1 (10^7 and 10^6 cells per ml) took up ^{35}S-labelled methionine at a much higher rate than did cells in freshly inoculated microcosms. Uptake was maximum for

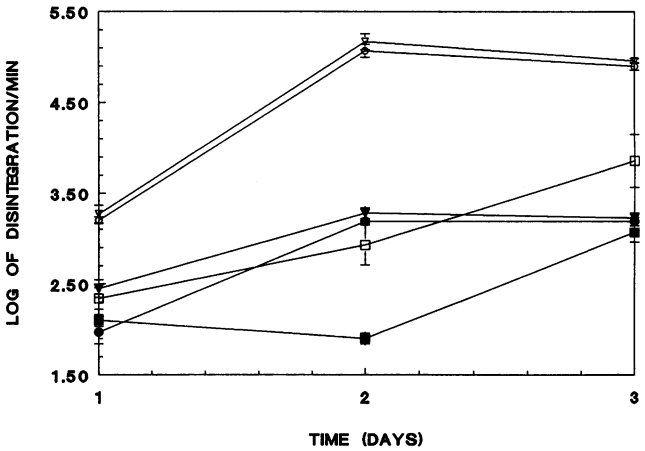


FIG. 3. Amino acid uptake assay of *S. dysenteriae* type 1 in culturable and VBNC states as a function of time and log of disintegrations per minute. Data for VBNC *S. dysenteriae* type 1 (10^7 cells per ml) 8 weeks after entering the VBNC state (○) and the same cells treated with 2% formalin (●), VBNC *S. dysenteriae* type 1 (10^6 cells per ml) 4 weeks after entering the VBNC state (▽) and the same cells treated with 2% formalin (▼), and cells from freshly inoculated microcosms (10^7 cells per ml) (□) and the same cells treated with 2% formalin (■) are shown. Data are means of duplicates, and error bars indicate the standard error for three trials.

VBNC *S. dysenteriae* type 1 but was less for freshly inoculated microcosms. The abiotic control was treated with 0.2% formalin before the addition of radioactively labelled methionine. Although formalin-treated cells appeared to take up a very small amount of methionine (Fig. 3), most likely reflecting a nonspecific binding of the amino acid by the cells, the methionine was not incorporated into cellular protein (Fig. 4, lanes

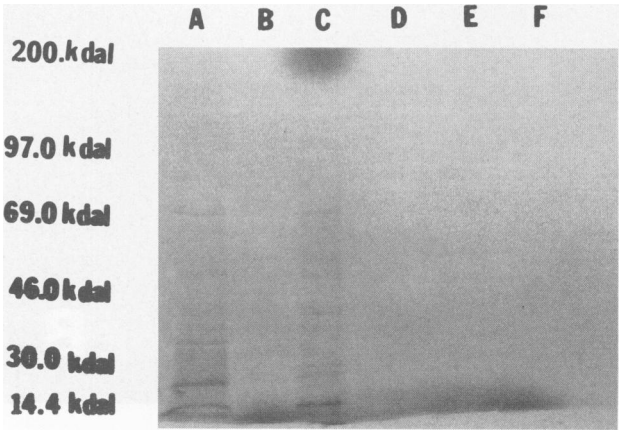


FIG. 4. Autoradiogram of VBNC *S. dysenteriae* type 1 labelled with [^{35}S]methionine following electrophoresis in a 15% SDS–polyacrylamide gel. Lanes: A, whole-cell lysate prepared from VBNC *S. dysenteriae* type 1 (10^7 cells per ml; 8 weeks after becoming VBNC); B, same as lane A, but treated with 2.0% formalin before the addition of the radiolabelled substrate; C, whole-cell lysate prepared from VBNC *S. dysenteriae* type 1 (10^6 cells per ml; 4 weeks after becoming VBNC); D, same as lane C, but treated with 2.0% formalin before the addition of the radiolabelled substrate; E, whole-cell lysate prepared from VBNC *S. dysenteriae* type 1 (10^7 cells per ml; freshly inoculated microcosm); F, same as lane E, but treated with 2.0% formalin before the addition of the radiolabelled substrate.

B and D).

On examination of the 3-day-average values, there were significant differences in the rates of uptake of labelled methionine in the three different microcosms, with and without formalin treatment. Essentially, values for the three treatments with formalin appeared the same, and all were lower than the value for the corresponding sample without formalin. There did not appear to be a statistically significant difference between samples differing only in VBNC cell concentration, whereas culturable cells did not take up labelled methionine as well, even when formalin was not present. The linear trend, i.e., the slopes, indicates that the uptake of labelled methionine by formalin-treated samples was not as rapid as it was for the corresponding nontreated samples. However, there did not appear to be a significant difference in the rates of uptake by VBNC cells and culturable cells over time.

The presence of significant quadratic trends indicates that the counts appear to increase initially and then decrease, or vice versa. The rate at which this occurs appears to differ among the microcosms. Rates of uptake of methionine by VBNC cells in the two microcosms containing VBNC cells (10^7 and 10^6 cells per ml) were approximately parallel in that both rose and fell at similar rates, whereas uptake by culturable cells was different in that little or no quadratic effect was observed, suggesting an increase in counts over time, with no significant decrease.

Resolution of whole-cell lysates by SDS-polyacrylamide gel analysis revealed that VBNC cells of *S. dysenteriae* type 1 incorporated labelled methionine (Fig. 4, lanes A and C) into protein. Lane E of Fig. 4 shows results for freshly inoculated microcosms, and lane F is the same sample treated with formalin. Although Fig. 3 shows methionine taken up by cells in freshly inoculated microcosms, data shown in Fig. 4 (lane E) suggest that it was not incorporated into protein.

DISCUSSION

This study was undertaken to examine the survival and viability of cells of *S. dysenteriae* type 1 subjected to severely nutrient-depleted conditions at selected temperatures, since nutrient depletion has been suggested to be one of the most common environmental parameters to which autochthonous and allochthonous organisms in the natural environment are routinely subjected (23). Previous studies in our laboratory showed that *S. dysenteriae* type 1 could persist in laboratory microcosms in a VBNC state for at least 6 months (17). In microcosms incubated at selected temperatures, cells were found to become nonculturable on routinely employed bacteriological media by days 12 and 21 at 30 and 37°C, respectively. These results are consistent with other reports in which investigators used stationary cultures at 25°C and employed pond, lake, river, and drain water to demonstrate the VBNC state for *S. dysenteriae* type 1 (10). However, results from our laboratory, as well as those from others, show that the time required to enter the VBNC state depends on several factors, including temperature (13), cell concentration, aeration (18), etc. The rate of nonculturability is different for each cell concentration; the reason for this was not investigated in this study. However, it is possible that at higher cell concentrations, organisms derive nutrients and energy from each other. Consistent with the findings of Linder and Oliver (13), size reduction during prolonged incubation was also observed in this study (Fig. 2A). This is believed to be a component of the survival strategy, i.e., bacterial size reduction to minimize energy requirements (16).

Methods used to monitor viability of the cells included the DVC procedure and uptake of INT. In the entire nonculturable population, it is very likely that a subset of cells become nonviable. One of the limitations of the current assays used for assessing viability of starved or VBNC cells is that it is extremely difficult to determine quantitatively the percentage of viable versus nonviable cells by microscopic analysis. Cephalexin was used in this study because the strain employed was resistant to nalidixic acid (11), recommended by Kogure et al. for the DVC procedure. Cephalexin (2) has been reported to demonstrate a mode of action similar to that of nalidixic acid, i.e., preventing cell division by inhibiting DNA gyrase, thereby terminating DNA polymerization and increasing cell permeability. Cephalexin has also been used by other investigators studying a strain of *E. coli* resistant to nalidixic acid (2). In addition to changes during transition to the VBNC state reported to date, there may be structural changes in the epitope on the outer surface, against which the rabbit polyclonal serum (used in the indirect immunofluorescent-antibody assay) was raised. Since VBNC *S. dysenteriae* type 1 was recognized, the conclusion is little or no change occurred, i.e., VBNC cells can be recognized serologically and this procedure, therefore, can be used for identification of *S. dysenteriae* type 1, whether culturable or nonculturable, in clinical samples. These data are in agreement with results reported by Islam et al. (10).

Maintenance of virulence by pathogens in the nonculturable state is controversial. Several authors have demonstrated that nonculturable cells of various pathogens, e.g., *E. coli*, *Yersinia enterocolitica*, *V. cholerae*, *Yersinia ruckeri*, and *Pasteurella piscicida*, retain pathogenicity. However, other investigators have reported loss of virulence by *V. vulnificus* and *A. salmonicida* in the nonculturable state (14). Chlorine- and copper-stressed *Y. enterocolitica* has been demonstrated to lose the ability to invade HeLa cells, while retaining infectivity, in mouse models (21). Hussong et al. reported that VBNC *L. pneumophila* retained pathogenicity for embryonated chick eggs (9). However, this result was reported several years ago, when studies of VBNC cells were relatively rudimentary. As such, criteria for the VBNC state were not well-defined. In addition, the fact that resuscitation was demonstrated for *V. vulnificus*, a phenomenon later found to be regrowth of a few culturable cells (24), signifies the need for redefining and standardizing the parameters and criteria to be met for the VBNC state. To date, there is no report of virulence of VBNC *S. dysenteriae* type 1 in either tissue culture experiments or animal models. It is interesting that, as shown in this study, VBNC *S. dysenteriae* type 1 retained cytopathic effect on HeLa cells. Since the VBNC cells were not screened for toxin production, the factor causing cytotoxicity in HeLa cells remains to be determined. However, VBNC cells may secrete a cytotoxic substance(s), e.g., Shiga toxin. Experiments in progress should clarify this point. Boiled cells were used as the negative control in this assay, since even trace amounts of formalin adversely affected the HeLa cells.

The uptake of amino acid by VBNC cells was a slow process, requiring incubation for at least 2 days before uptake was measurable (Fig. 4). However, the uptake rate was higher than that of fresh cells. The reason for this is not yet known. Results of this study do not indicate whether uptake is by active transport or diffusion, and either one or a combination of both may occur. It appears that fresh cells are not capable of taking up low concentrations of amino acids, perhaps because fresh cells are shocked when transferred to a nutrient-depleted medium. Incorporation of ^{35}S-labelled methionine by VBNC *S. dysenteriae* type 1 was measured by using whole-cell lysates prepared from each sample that were collected at 24-h inter-

vals. Samples showing the highest level of radioactivity (day 2) were analyzed by SDS-polyacrylamide gel analysis. Results showed that methionine was actively taken up by VBNC cells and incorporated into protein. In addition to the DVC and INT-DVC results, the methionine uptake assay, with SDS-polyacrylamide gel analysis, clearly substantiated the presence of continued respiration and protein synthesis in the VBNC cells. Stable enzymes with a long half-life and activity sufficient to incorporate amino acids but not to allow for cell replication may be an explanation, but no experimental evidence has been provided to support such an hypothesis.

This is the first report showing VBNC cells of *S. dysenteriae* type 1 to be both metabolically active and cytopathogenic for HeLa cells. VBNC pathogens will pose a considerable threat to public health if it can be unequivocably established that they, indeed, retain virulence. However, even if cells are not virulent in the VBNC state, the cells may be capable of virulence after regaining culturability. It is important that VBNC cells have been shown to maintain plasmids (3). The question of virulence is more complicated for *S. dysenteriae* type 1, since virulence is both plasmid and chromosomally mediated (7). Nevertheless, on the basis of results of this study, regulation of genes responsible for proteins that are turned on or off in response to loss of culturability is significant, and this phenomenon is the object of further study in our laboratory. To date, regulation of metabolism in cells has been shown to be altered under conditions of starvation, but such cells are still culturable (13, 23). Those mechanisms underlying the phenomenon of loss of culturability are intriguing, especially in relation to pathogenicity. Thus, this serves as a suitable target for future study.

ACKNOWLEDGMENTS

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REFERENCES

1. **American Public Health Association.** 1981. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
2. **Boye, E., and A. Lobner-Olesen.** 1991. Bacterial growth control studied by flow cytometry. Res. Microbiol. **142**:131–135.
3. **Byrd, J. J., and R. R. Colwell.** 1990. Maintenance of plasmids pBR322 and pUC8 in nonculturable *Escherichia coli* in the marine environment. Appl. Environ. Microbiol. **56**:2104–2107.
4. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but nonculturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3**:817–820.
5. **Colwell, R. R., M. L. Tamplin, P. R. Brayton, A. L. Tavgens, B. D. Tall, D. Herrington, M. M. Levine, S. Hall, A. Huq, and D. A. Sack.** 1990. Environmental aspects of *Vibrio cholerae* in transmission of cholera, p. 327–343. *In* R. B. Sack and R. Zinaki (ed.), Advances in research on cholera and related areas, 7th ed. KTK Scientific Publishers, Tokyo.
6. **Draper, N. R., and H. Smith.** 1981. Applied regression analysis, 2nd ed. Wiley & Sons, New York.
7. **Hale, T. L.** 1991. Genetic basis of virulence in *Shigella* species. Microbiol. Rev. **55**:206–224.
8. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
9. **Hussong, D., R. R. Colwell, M. O'Brian, A. D. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar. Media Biotechnol. **5**:947–950.
10. **Islam, M. S., M. K. Hasan, M. A. Miah, G. C. Sur, A. Felenstein, M. Venketesan, R. B. Sack, and J. Albert.** 1993. Use of the polymerase chain reaction and fluorescent-antibody methods for detecting viable but nonculturable *Shigella dysenteriae* type 1 in laboratory microcosms. Appl. Environ. Microbiol. **59**:536–540.
11. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Linder, K., and J. D. Oliver.** 1989. Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl. Environ. Microbiol. **55**:2837–2842.
14. **Magarinos, B., J. L. Romalade, J. L. Barja, and A. E. Toranzo.** 1994. Evidence of a dormant but infective state of the fish pathogen *Pasteurella piscicida* in seawater and sediment. Appl. Environ. Microbiol. **60**:180–186.
15. **Nilsson, L., J. D. Oliver, and S. Kjelleberg.** 1991. Resuscitation of *Vibrio vulnificus* from the viable nonculturable state. J. Bacteriol. **173**:5054–5059.
16. **Postgate, J. R.** 1976. Death in macrobes and microbes. Symp. Soc. Gen. Microbiol. **26**:1–18.
17. **Rahman, I., M. Shahamat, and R. R. Colwell.** 1993. Comparison of protein profiles of *Shigella dysenteriae* type 1 in culturable and nonculturable states, abstr. Q-4, p. 11. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993.
18. **Rollins, D. M., and R. R. Colwell.** 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the aquatic environment. Appl. Environ. Microbiol. **52**:531–538.
19. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51**:365–379.
20. **Shahamat, M., U. Mai, C. Paszko-Kolva, M. Kessel, and R. R. Colwell.** 1993. Use of autoradiography to assess viability of *Helicobacter pylori* in water. Appl. Environ. Microbiol. **59**:1231–1235.
21. **Singh, A., and G. A. McFeters.** 1987. Survival and virulence of copper- and chlorine-stressed *Yersinia enterocolitica* in experimentally infected mice. Appl. Environ. Microbiol. **53**:1768–1774.
22. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry, 2nd ed. W. H. Freeman and Co., New York.
23. **Tempest, D. H., and O. M. Neijssel.** 1981. *In* A. Hollaender (ed.), Basic life sciences, vol. 18, p. 335–356. Plenum Press, New York.
24. **Weichert, D., J. D. Oliver, and S. Kjelleberg.** 1992. Low temperature induced nonculturability and killing of *Vibrio vulnificus*. FEMS Microbiol. Lett. **100**:205–210.
25. **Zimmerman, R., R. Iturriaga, and J. Becker-Birch.** 1979. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.