Induction and Resuscitation of Viable but Nonculturable *Salmonella enterica* Serovar Typhimurium DT104†

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Salmonella enterica serovar Typhimurium DT104 11601 was tested for its ability to maintain viability in minimal, chemically defined solutions. Periodic monitoring of growth and survival in microcosms of different ion concentrations, maintained at various temperatures, showed a gradual decline in culturable organisms (~235 days) at 5°C. Organisms maintained at a higher temperature (21°C) showed continuous, equivalent CFU per milliliter (~10⁶) up to 400 days after inoculation. Fluorescence microscopy with BacLight revealed that nonculturable cells were actually viable, while observations with scanning electron microscopy showed that the cells had retained their structural integrity. Temperature upshift (56°C ± 0.5, 15 s) of the nonculturable organisms (5°C) in Trypticase soy broth followed by immediate inoculation onto Trypticase soy agar (TSA) gave evidence of resuscitation. Interestingly, *S. enterica* serovar Typhimurium DT104 from the microcosms at either 5°C (1 to 200 days) or 21°C (1 to 250 days) did not show enhanced growth after intermittent inoculation onto catalase-supplemented TSA. Furthermore, cells from 21°C microcosms exposed to oxidative and osmotic stress showed greater resistance to stresses over increasing times of exposure than did recently grown cells. It is possible that the exceptional survivability and resilience of this particular strain may in part reflect the growing importance of this multidrug-resistant organism, in general, as a cause of intestinal disease in humans. The fact that *S. enterica* serovar Typhimurium DT104 11601 is capable of modifying its physiological characteristics, including entry into and recovery from the viable but nonculturable state, suggests the overall possibility that *S. enterica* serovar Typhimurium DT104 may be able to respond uniquely to various adverse environmental conditions.

Numerous members of the genus *Salmonella* are recognized internationally as major foodborne pathogens. Foodnet reports implicate salmonellosis as the number one infectious disease in the United States (4). Although the mortality rate resulting from salmonellosis is low, costs associated with *Salmonella* infection range from \$983 million to \$1.4 billion (10). *Salmonella* is responsible for gastroenteritis and septicemia, with complications resulting in meningitis, cholecystitis, and peritonitis. The Centers for Disease Control and Prevention reported that DT104 was identified in 32% of the *Salmonella enterica* serovar Typhimurium strains isolated from humans in 1996, an increase from 28% in 1995 and 7% in 1990. DT104 is now only the second most prevalent type of *Salmonella* after serovar Enteritidis phage type 4 in the United Kingdom (9). *S. enterica* serovar Typhimurium DT104 is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (2), and more recently, resistance has been shown to trimethoprim and ciprofloxacin (18). Multiantibiotic resistance provides this organism with a survival advantage, thus hindering successful treatment of infections.

Additionally, salmonellae have several other modes of survival, including formation of biofilms, resistance to low water activity (7), rugose formation (1), and entry into a viable but

nonculturable (VBNC) state (5). The VBNC phenomenon has been previously described in numerous human pathogens, including *Escherichia coli* (32), *Salmonella enterica* serovar Enteritidis (27), *Vibrio cholerae* (6), *Vibrio vulnificus* (22), and *Campylobacter jejuni* (25, 29).

VBNC denotes a state in which the cells cannot be detected by standard culture on enriched agar media, although remaining viable and capable of resuscitation under favorable conditions. Employed mainly by gram-negative bacteria, this state has been proposed as a strategy for survival by bacteria in the natural environment (26). Temperature (22), as well as nutrient limitation (12, 13), levels of osmolarity (27), humidity (8), and aeration (25), has been found to be important factors that contribute to the induction of the VBNC state. In the absence of growth, various viability assays have been successfully employed to detect the presence of VBNC cells, such as direct viable count (14); fluorescence microscopy using various stains, e.g., cyanoditotyl tetrazolium chloride (CTC) (24); and more recently molecular genetic methods, e.g., PCR (31) and gel electrophoresis (15). However, the eventual proof of vitality is through resuscitation of the cells and confirmation that the recovered cells were indeed identical to the starting cells.

In some of our previous studies (23), there have been indications of a possible VBNC state in *Salmonella* that could account for poultry broiler farms testing either positive or negative for *Salmonella* at different times depending upon the amount of water activity (*A_w*) in the litter. If this were the case, the *Salmonella* might still be capable of infectivity, as previously shown with *V. cholerae* (6), if carried by poultry at market and ingested by susceptible, human hosts. To determine the kinetics of the entry of *S. enterica* serovar Typhimurium DT104

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into the VBNC state and its eventual resuscitation in this study, the organism was inoculated into static, nutrient-deficient microcosms for more than a year with successful demonstration that the organism could become VBNC. While resuscitation has been accomplished with organisms in the genus *Vibrio*, the genetic and physiologic mechanisms that control this reversion have still not been sufficiently described. Using specific temperature upshifts, we have been successful, for the first time, in resuscitating *S. enterica* serovar Typhimurium DT104.

Entry of *S. enterica* serovar Typhimurium DT104 into the VBNC state is temperature dependent, suggesting a process similar to that of some marine vibrios, wherein the VBNC response at low temperatures (22) differs from the starvation response at higher temperatures. Even though *S. enterica* serovar Typhimurium DT104 has been implicated in several outbreaks and has a multidrug-resistant pattern, very few studies have attempted to identify the ability of this organism to respond to various environmental stresses. Herein, we report the variable responses exhibited by *S. enterica* serovar Typhimurium DT104 11601 to different temperatures and while maintained under nutrient deprivation, as well as the interaction among starvation survival, cross-protection, and entry into and resuscitation from the VBNC state.

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MATERIALS AND METHODS

Media and growth conditions. *S. enterica* serovar Typhimurium DT104 strain 11601(a kind gift from B. Swaminathan) was cultured on Trypticase soy agar (TSA) (Difco, Detroit, Mich.) and incubated at 37°C. Growth on xylose-lysine-tergitol-4 (XLT4) (Difco) was used to identify presumptively the presence of *Salmonella*. Cells were grown statically for 24 h at 21°C in 10 ml of Trypticase soy broth (TSB) (Difco) in glass test tubes (17 × 150 mm). The cells were washed and resuspended in phosphate-buffered saline (PBS) to a final concentration of $\sim 10^8$ CFU/ml. From this suspension, 8.0 ml was transferred to 500-ml glass flasks (microcosms) containing 400 ml each of 7.35, 0.735, and 0.0735 mM Butterfield phosphate solution (BPS), respectively (7a). Microcosms with no inoculum were used as controls (5) to monitor continuous sterility of the microcosm medium. Three inoculated and control microcosms each were incubated at 5 and 21°C and monitored by standard heterotrophic plate counts on TSA (Difco) and by fluorescence microscopy. Sampling was done with sterile pipettes after carefully flaming the tops of the flasks. Before removal of the inoculum dose, the flask was gently rotated. Confirmation of *S. enterica* serovar Typhimurium DT104 from the cultures was accomplished by using biochemical tests, including triple sugar iron agar slants (TSI) (Difco) and API 20E strips (Bio-Merieux, Inc.), flagellar broth, and subsequent serological reactions with the somatic (O) and flagellar (H) antigens (Difco).

Determination of viability by fluorescence microscopy. Qualitative analysis of the presence of culturable cells was performed with the fluorescent LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, Oreg.) and CTC (Polysciences, Warrington, Pa.). Briefly, samples from the microcosm suspensions were stained with CTC to detect respiratory activity, as described by Rodriguez et al. (24). CTC was diluted in distilled water to a final concentration of 5 mM, and the stained suspension was incubated for 4 h at 37°C. BacLight was used according to the manufacturer's recommended protocol to differentiate between live and dead cells. The stained suspension was incubated at 21°C for 15 min, and a 10- μ l aliquot was placed on poly-L-lysine-coated slides (Sigma) and visualized by using a Zeiss fluorescence microscope (Zeiss III RS-West Germany). The total cell population was counted by grid motion and multiplied by 100 to obtain the number of CFU per milliliter. Formalin (Sigma)-treated cultures were used as controls in the fluorescence microscopy experiments.

Determination of ultrastructure integrity. For observation of morphology and to determine the size of the starved organisms, 1.0-ml suspensions from microcosms maintained at 5 and 21°C for more than 365 days and from fresh cultures were processed for scanning electron microscopy (SEM). Briefly, cell suspen-

sions were fixed in 2% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide, dehydrated with graded concentrations of ethanol, critical point dried, and coated with gold-palladium alloy. Samples were examined with an Amray 1820 D scanning electron microscope (Amray, Inc., Bedford, Mass.).

Determination of resuscitation. (i) **Temperature upshift.** Attempts to resuscitate the organism when 10 ml of the 7.35 mM suspension maintained at 5°C was plated in 1.0-ml aliquots on 10 different TSA plates, followed by incubation at 37°C for 24 h, failed to reveal culturable cells. In a follow-up experiment, equal volumes (0.5 ml) of the nutrient-deprived culture and TSB in a Durex, borosilicate, 13-by-100-mm glass tube were immersed in a preheated 80°C water bath for 15 s, allowing the temperature of the internal contents to reach 56°C $\pm$ 0.5. Then, two 100- μ l samples were cultured on TSA plates and incubated at 37°C.

(ii) **Quantitative measurement.** Four 5.0-ml aliquots (10^5 CFU/ml each) were aseptically removed from the 7.35 mM microcosm maintained at 5°C at day 273 and were mixed separately with equal volumes of TSB, yielding 5×10^4 CFU/ml in each of four 15-ml test tubes. The test tubes were incubated at either 5, 21, 25, or 37°C, respectively, for 18 h. One hundred-microliter aliquots were then monitored every hour by performing standard serial heterotrophic plate counts.

Susceptibility of nutrient-deprived cells to oxidative and osmotic stresses. To assay resistance to oxidative stress in nutrient-limited organisms, a 1.0-ml sample from the 21°C, 7.35 mM microcosm suspension from day 418 was mixed with 9.0 ml of TSB, yielding a suspension of $\sim 10^4$ CFU/ml. A 24-h culture grown statically in TSB at 21°C was used as the control. Hydrogen peroxide was added to yield a final concentration of 10 mM. Samples were monitored up to 100 min at 37°C by recovering 100- μ l aliquots and performing standard dilutions and replicate plate count assays on TSA for 24 h at 37°C.

To assay for osmotic stress, replicate 1.0-ml samples from the 7.35 mM microcosm suspensions from day 178 and maintained at 5 and 21°C were mixed with 9.0 ml each of saturated NaCl solution made with glass-distilled water and yielding a suspension of $\sim 3 \times 10^4$ CFU/ml. The samples were incubated from 24 to 168 h at 21°C. Then, 100- μ l aliquots were removed and quantified as described above. Nonstressed cultures grown at 21°C were included as controls.

Evaluation of the effect of catalase on resuscitation of VBNC *S. enterica* Typhimurium DT104. *Aspergillus niger* catalase (Sigma, St. Louis, Mo.) was added to pre-poured TSA agar plates to a final concentration of 2,000 IU per plate. Nonsupplemented TSA plates were used as controls. Using a separate set of microcosms containing 2.3% Instant Ocean (Aquarium Systems, Mentor, Ohio) and maintained at different temperatures (5, 21, and 37°C) up to 240 days, 100- μ l aliquots were removed at periodic intervals, diluted, and inoculated onto catalase-supplemented and nonsupplemented TSA plates by using the standard serial plate count procedure followed by incubation at 37°C for 24 h to determine the comparative effect, if any, of catalase on the recovered population sizes. A change of medium from BPS to Instant Ocean was made, because it had been observed that vibrios exposed to Instant Ocean had become nonculturable in a much shorter time than with other solutions containing buffered salts.

RESULTS

Culturability of *S. enterica* serovar Typhimurium DT104 after long-term nutrient starvation. The heterotrophic plate count of organisms maintained at 5°C at the three concentrations of BPS showed a gradual decrease in numbers from $\sim 10^6$ to $\sim 10^3$ CFU/ml at ~ 180 days, eventually declining to 10^0 CFU/ml at about 273 days (Fig. 1). The results for the 0.735 mM BPS experiments are not depicted in Fig. 1. Results similar to those noted with the other concentrations (0.0735 and 7.35 mM BPS) were observed in the 0.735 mM BPS microcosms. The organisms starved at 21°C in three different buffer concentrations did not show any significant decrease in culturability even after a nutrient limiting period of more than 365 days. For the microcosms maintained at 5°C, the viable cell count, determined by fluorescence microscopy using BacLight and CTC, revealed counts between 1,000 and 1,300 live cells per ml even when the plate count did not reveal any colonies: e.g., a 10-ml suspension from the starved microcosm inoculated in 1.0-ml amounts on 10 separate TSA plates failed to yield even a single colony after incubation at 37°C.

FIG. 1. Effect of nutrient limitation on *S. enterica* serovar Typhimurium DT104 11601 maintained in microcosms at 21°C (full line) containing 7.35 mM (●) and 0.0735 mM BPS and at 5°C (broken line) in 7.35 (□) and 0.0735 (○) mM BPS over 425 days. Each data point represents a single measurement of the number of CFU per milliliter. Comparable results were obtained with microcosms containing 0.735 mM BPS when the cells were maintained at 5 and 21°C and held for the same period of time (data not shown). Points shown on the baseline represent undetectable populations after standard plate count determinations.

Determination of ultrastructural integrity. Organisms starved for nutrients and maintained at 21°C for >365 days exhibited structural integrity and a significant increase in the number of fibrils observed (Fig. 2A). In contrast, cells freshly cultured at 21°C for 24 h did not show an increase in the number of fibrils (Fig. 2B). Interestingly, some of the organisms starved at 5°C for the same period of time exhibited a gradual rounding of the cells—i.e., conversion from a bacillary to a coccobacillary shape—but appeared to maintain their structural integrity (Fig. 3).

Tolerance of starved organisms to hyperosmotic and oxidative stress. *Salmonella* spp. are known to develop resistance to hyperosmotic stress, and this response is amplified by nutrient limitation (11). In this study, development of resistance to osmotic stress appeared to be relatively higher only in starved organisms that had been previously maintained at 21°C than those maintained at either 5°C or those recently cultured at

FIG. 3. Scanning electron micrograph of representative *S. enterica* serovar Typhimurium DT104 11601 exhibiting bacillary and coccoid cell morphologies after exposure to nutrient-limiting conditions for more than 365 days. Scale bar, 1 μ m.

21°C (Fig. 4). In comparison with the nonstressed, recently cultured cells, those from 5°C microcosms appeared to be more resistant after ~60 h of osmotic stress, but resistance declined precipitously after further exposure. In contrast, cells from the 21°C microcosms showed consistently greater resistance to hyperosmotic conditions. Similarly, greater protection was observed in organisms maintained in 21°C microcosms that were eventually exposed to 10 mM H_2O_2 , whereas freshly

FIG. 2. Scanning electron micrographs of *S. enterica* serovar Typhimurium DT104 11601 exposed to nutrient-limiting conditions for more than 365 days (A) versus cells grown for 24 h in TSB (B). Scale bar, 1 μ m.

FIG. 4. Comparison of the survival of a freshly cultured suspension ($\sim 10^4$ CFU/ml) (▲) and a nutrient-deficient suspension of *S. enterica* serovar Typhimurium DT104 11601 maintained in 7.35 mM BPS microcosms at 21°C (■) and 5°C (○) for 178 days after exposure to saturated NaCl for 22 to 168 h. Each data point represents a single measurement of the number of CFU per milliliter.

cultured organisms showed less resistance to the oxidant, particularly at 40 and 60 min (Fig. 5).

Resuscitation of VBNC cells. Organisms maintained at 5°C were observed to undergo an “apparent die-off” between 250 to 300 days when cultured on TSA (Fig. 1). In a follow-up experiment, the nonculturable organisms, after exposure to $56 \pm 0.5^\circ\text{C}$ for 15 s and inoculation onto TSA, presented as an uncountable number of colonies on the plate (Fig. 6). This phenomenon was observed repeatedly for various exposure time points at $56 \pm 0.5^\circ\text{C}$ from 5 to 30 s. To determine the status of these cells (i.e., dead versus VBNC), organisms from the 7.35 mM BPS microcosms maintained at 5°C were exposed to a temperature upshift (37°C) in TSB and monitored hourly until the tubes became highly turbid, indicating elevated cell growth at 18 h. After a considerable lag phase of approximately 10 h, there was a sudden appearance of culturable organisms ($\sim 10^3$ CFU/ml) in a period of 1 h, or possibly less (Fig. 7), only from the suspension that had been exposed to a temperature upshift to 37°C but not from the suspensions that had been incubated at 5 and 21°C . At 11 to 14 h of incubation, a second slope suggested a combination of resuscitation and multiplication, reaching a level of $\sim 10^5$ CFU, and at 15 to 17 h of

incubation, there was a third slope, suggesting exponential growth of the organisms up to $\sim 10^8$ CFU. The beginning of a plateau at approximately 18 h of incubation suggested a stationary phase (Fig. 7). The same resuscitation phenomenon involving an extended time lag and sudden appearance of viable organisms was observed in duplicate experiments with *S. enterica* serovar Typhimurium DT104.

Effect of catalase on resuscitation of nonculturable cells. To consider the possibility that the organisms in the microcosms were becoming VBNC after exposure to oxidants in their environment, the effect of an antioxidant, catalase, was evaluated periodically, while attempting to recover cells maintained in the microcosms up to 240 days by standard plate count. Because of previous reports that described enhancement of growth of VBNC *V. cholerae* by addition of catalase to the agar medium prior to culture, an attempt was made to determine if similar effects applied to *S. enterica* serovar Typhimurium DT104 as well. *S. enterica* serovar Typhimurium DT104 from 5 and 21°C microcosms inoculated and quantified on TSA supplemented with *A. niger* catalase (2,000 IU) (Sigma) at different time intervals (1 to 240 days) failed to show any increase in the culturability of the organisms in comparison to cultures on TSA without supplementation (Fig. 8). Cells maintained at 5°C were completely nonculturable on TSA agar at 200 days and afterwards with and without supplemented catalase.

DISCUSSION

This study shows for the first time the development of the VBNC state in *S. enterica* serovar Typhimurium DT104 after exposure to low temperatures in nutrient-limiting microcosms for prolonged periods of time. It was possible to resuscitate the nonculturable organisms by temperature upshift and nutrient addition. Very importantly, such resuscitation could be discerned and differentiated from simple multiplication by periodic monitoring of the culturable count among the VBNC organisms after temperature upshift (37°C) and exposure to a nutrient-rich broth. Furthermore, catalase-supplemented TSA was not beneficial for the resuscitation of VBNC *S. enterica* serovar Typhimurium DT104. The nonculturable response at 5°C for 273 to 300 days was different from the starvation response exhibited when the cells were maintained at 21°C for the same time period. The organisms maintained at 21°C maintained culturability for the entire period of the study, which was perhaps due to increased starvation at 21°C that inhibits the VBNC response as suggested by Oliver et al. (22).

Starvation response has been shown to render cross protection to osmotic challenge in *E. coli* (11). Formation of unique starvation proteins appears to be responsible for this phenomenon (11), which may also explain the increased survivability of similarly challenged *S. enterica* serovar Typhimurium DT104. Indeed, *S. enterica* serovar Typhimurium DT104 grown at 21°C in a nutrient-limited environment appeared to be more capable of resisting unfavorable conditions such as oxidation and osmolarity. A proteomic analysis of stressed *S. enterica* serovar Typhimurium DT104 could possibly reveal novel stress proteins that haven't been recognized previously. We observed the connection between cross-protection and starvation survival mechanisms at 25°C but did not observe the VBNC phenomenon, again indicating that the same organism can exhibit two

FIG. 5. Comparison of the survival of freshly cultured (gray bars) ($\sim 3 \times 10^4$ CFU/ml) and nutrient-deficient *S. enterica* serovar Typhimurium DT104 11601 ($\sim 3 \times 10^4$ CFU/ml) (black bars) maintained in 7.35 mM BPS microcosms at 21°C for 418 days, after exposure to 10 mM H_2O_2 solution for 100 min. Counts represent the mean of replicate experiments. Points shown on the baseline represent undetectable populations after standard plate count determinations.

FIG. 6. Comparison of nonculturable *S. enterica* serovar Typhimurium DT104 11601 ($\sim 5 \times 10^4$ CFU/ml) exposed to an 80°C (56°C internal temperature) upshift for 15 s (left) versus no temperature upshift (right) followed by inoculation on TSA at 37°C for 24 h. Selected colonies from the left plate were tested with Gram stain, TSI agar slants, and API 20E and were confirmed to be *Salmonella*.

different responses: i.e., starvation survival at 21°C and VBNC at 5°C.

Previous research in our laboratory had shown a decrease in culturability of *Salmonella* spp. in response to decreased levels of Aw, suggesting that cells were possibly entering a nonculturable state (7). The presence of live cells in the absence of culturability was confirmed and extensive filamentation in *S. enterica* serovar Typhimurium DT104 after exposure to low Aw was revealed by using BacLight and CTC (7).

FIG. 7. Resuscitation of nonculturable *S. enterica* serovar Typhimurium DT104 11601 maintained in 7.35 mM BPS at 5°C for 273 days when 5.0 ml of a concentration of $\sim 10^5$ CFU/ml was added to 5.0 ml of brain heart infusion broth (final concentration, 5×10^4 CFU/ml) and subjected to temperature upshifts to 21°C (X), 25°C (■), and 37°C (●). Cultures maintained at 5°C (◆) without temperature upshift served as controls. Counts represent the mean of replicates. Except for the cultures subjected to a 37°C upshift, the timelines for the other cultures remained on the baseline. The points shown on the baseline represent undetectable populations after standard plate count determinations.

SEM of our nutritionally stressed but viable cells showed the presence of increased numbers of fibrils (Fig. 2A) and gradual rounding of the cells (Fig. 3). This change in morphology from a bacillary to a coccobacillary or even coccoid form has been previously observed in *Vibrio* spp. (20) and *Campylobacter* spp. (25), along with changes in membrane composition (16). As observed previously with other VBNC cells, *S. enterica* serovar Typhimurium DT104 appeared to maintain gross membrane integrity after prolonged maintenance in nutrient-deficient microcosms (17) when examined by SEM (Fig. 3).

FIG. 8. Comparison of the recovery of nutrient-deprived *S. enterica* serovar Typhimurium DT104 11601 previously maintained in microcosms containing 2.3% Instant Ocean at 5°C (○) and 21°C (▲) on catalase-supplemented (broken line) versus nonsupplemented (solid line) TSA plates. Comparable results were obtained with similar microcosms that had been maintained at 37°C for the same period of time. Points shown on the baseline represent undetectable populations after standard plate count determinations.

The time required for the nutrient-limited *S. enterica* serovar Typhimurium DT104 11601 cells to become nonculturable was greater than had been observed previously with other gram-negative enteric organisms. These observations combined with the fact that *S. enterica* serovar Typhimurium DT104 is resistant to most of the conventional antibiotics and can attain a rugose morphology (1, 2) suggest that *S. enterica* Typhimurium DT104, in general, may be a highly resilient organism.

Although previous studies have shown that some enteric pathogens are capable of resuscitating and growing to large populations (18–20), others have suggested that this observation occurs because of the regrowth of a few surviving cells (12). A *V. cholerae* O1 strain had been successfully resuscitated into a lawn of bacteria by subjecting nonculturable cells to a 45°C temperature upshift (30). Our nonculturable organisms failed to resuscitate when subjected to similar treatment (data not shown). Interestingly, nonculturable *S. enterica* serovar Typhimurium DT104 when exposed to a temperature upshift to $56 \pm 0.5^\circ\text{C}$ for various, short periods of time did result in the appearance of a large number of colonies following incubation at 37°C. This observation strongly suggested that there were large numbers of existing, viable *S. enterica* Typhimurium DT104 in the microcosm, thus supporting the hypothesis that the organism enters the VBNC state. The finding of recoverability of previously VBNC *S. enterica* Typhimurium DT104 appears convincing, because such a rapid appearance in the number of cells could not have happened by simple multiplication alone. Moreover, maintenance in low-nutrient medium may be responsible for synthesis of novel stress proteins. In fact, 100 μl of the 5°C microcosms maintained for 273 days yielded uncountable numbers of colonies after exposure to a $56 \pm 0.5^\circ\text{C}$ upshift for 15 s. These numbers were seemingly greater than the population counted in the quantitative and fluorescent microscopy studies, perhaps reflecting the normal variation of dormant (apparently nonviable) cells exposed to stressful conditions, which may have undergone a sudden resurgence of their metabolism after exposure to a high and sudden rise in temperature.

When nonculturable cells from 5°C microcosms were incubated in TSB at 37°C and monitored hourly, there was an 11-h latent phase followed by a 3-log increase in cell population in 1 h or less. Clearly, this significant increase in population indicated a reemergence of nonculturable cells.

The VBNC phenomenon has been attributed to the inability of nutritionally deprived cells to adapt to a catalase-deficient agar medium (3). To address the possibility that resuscitation did not occur after direct inoculation on TSA plates because of a requirement for more reduced conditions, cells from the microcosms were periodically inoculated for 240 days on catalase-supplemented and nonsupplemented TSA plates. It has been suggested that addition of catalase to the agar can successfully resuscitate presumed VBNC *V. cholerae* that had become sensitive to the generation of reactive oxygen species (3). Experiments performed to test the effect of catalase on resuscitation of VBNC *S. enterica* Typhimurium DT104 showed that the organism did not appear to be influenced by the addition of catalase to the medium. Basically there was no significant difference in the number of culturable cells between the non-supplemented controls and the catalase-supplemented test plates cultured at 37°C. Similar to the pattern observed in the

survival study (Fig. 1), the *S. enterica* serovar Typhimurium DT104 cells maintained at 5°C and nonrecoverable after 180 days were not enhanced in the presence of catalase (Fig. 8). In fact, resuscitation was observed to be diminished over prolonged maintenance at the same time point under each condition. Since catalase did not improve the culturability of cells, it is probable that there was no hydrogen peroxide sensitivity associated with the cells that had been presumed to be VBNC.

Microorganisms are constantly exposed to nutrient deprivation and a variety of stresses that must be overcome for successful propagation of the species. Various physiological changes like size reduction (25), changes in protein synthesis (21), decreased metabolic activity (19), and cell wall modifications (28) aid in successful survivability. Using germination following sporulation as an analogy, it is possible that VBNC may be another response that enables organisms to maintain viability, eventually reverting back to the normal state after a return of favorable conditions in the environment. When the environmental signals responsible for stimulating entry of *S. enterica* serovar Typhimurium DT104 into the VBNC state and the internal signaling pathways responsible for loss and gain of culturability are eventually determined, the probabilities for developing interventions against *S. enterica* serovar Typhimurium DT104 in environments such as those associated with poultry production and processing will be much improved.

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