Recovery of Culturability of an HOCl-Stressed Population of *Escherichia coli* after Incubation in Phosphate Buffer: Resuscitation or Regrowth?

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An *Escherichia coli* population harvested in exponential phase at about 10^8 cells/ml was treated in phosphate buffer with HOCl at concentrations ranging from 0.4 to 1 mg/liter (7.7 to 19 μM). The HOCl stress resulted in the appearance of three cell subpopulations: a majority of dead (nonrespiring) cells, a few culturable cells (10^2 to 10^4), and about 10^7 viable but nonculturable cells. In the absence of any added exogenous nutrient, a culturable population could be recovered after 1 day of incubation in phosphate buffer, and such a population would reach a cell density close to 10% of the initial density of the stressed population, whatever the initial number of survivors. When a small number of untreated cells were mixed with the stressed population, growth of the untreated cells was observed, demonstrating that damaged cells provided nutrients. Similarly, a filtrate and a disrupted-cell filtrate of the stressed population supported growth of untreated cells with the same efficiency. The number of CFU (untreated or stressed) at plateau phase depended on the initial density of the stressed cells. Taken together, these results suggest that recovery in phosphate buffer of an HOCl-stressed population is in large part due to growth of a few culturable cells at the expense of damaged cells. However, comparison of the growth rates of the stressed culturable population and of untreated bacteria growing in filtrate showed significantly faster growth of the stressed cells, a fact not fully compatible with the hypothesis that recovery is only the simple growth of survivors. We suggest, therefore, that in addition to growth of the few culturable stressed cells, there is repair and growth of some mildly injured viable but nonculturable cells.

Chlorination is the most widely used method of disinfecting water. The effectiveness of disinfection is commonly determined by monitoring culturable coliform bacteria. However, the absence of culturable bacteria may not adequately reflect the state of the original population. For example, after exposure to hypochlorous acid (HOCl), an *Escherichia coli* K-12 population will form three subpopulations (10): (i) culturable bacteria, which are able to form colonies on solid medium; (ii) viable but nonculturable (VBNC) bacteria, which are unable to form colonies but which still display respiratory or metabolic activity detectable by direct viability assays such as CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (26) and INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride] assays (35) or by direct viable count (13) tests; and (iii) dead bacteria.

Numerous bacteria, such as *E. coli*, *Vibrio cholerae*, *Vibrio vulnificus*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, and *Campylobacter jejuni* (2, 3), can enter the VBNC state after exposure to adverse environmental conditions. Therefore, it is important to know the physiological capacities of bacteria once they have entered the state of nonculturability. Are there conditions which will enable these bacteria to multiply and recover pathogenicity?

V. vulnificus becomes nonculturable under starvation conditions when incubated at a low temperature in an artificial seawater microcosm or estuarine seawater (21, 22). An upshift to room temperature, without addition of nutrients, leads to a recovery of culturability. Whether the increase in the number

of CFU results from true resuscitation of VBNC cells or from regrowth of a few culturable cells at the expense of dead or moribund cells is controversial (23). Findings from earlier studies suggested that cells underwent resuscitation (21), whereas recent studies led to the conclusion that the culturability was due to regrowth of a small culturable fraction (8, 31, 32). Similar results were obtained with *V. cholerae* (25) and *Vibrio parahaemolyticus* (11). Nonetheless, VBNC cells of *V. vulnificus* undergo changes at low temperatures (15) which potentially allow them to persist for extended periods (31), and there may be particular conditions which lead to true resuscitation. Indeed, a large fraction of cells from *V. cholerae* TSI-4 which had entered the VBNC state after 100 days' incubation at 15°C in M9 salt solution containing ammonium salts recovered culturability after a 1-min heat shock at 45°C. This recovery occurred only in M9 and not in solutions that lack ammonium, such as phosphate-buffered saline (30). In experiments testing the culturability of *Micrococcus luteus*, cells which had been starved for several months showed an extremely low degree of culturability and were not resuscitable when diluted in lactate growth medium. Strikingly, the cells recovered culturability when supernatants from stationary-phase cultures were added to the growth medium, suggesting that viable cells produce a factor which stimulates resuscitation (12, 29). Recently, Whitesides and Oliver presented data for a model that supports a true resuscitation of VBNC *V. vulnificus* cells following a temperature upshift. This resuscitation was not observed in the presence of elevated nutrient levels, suggesting some inhibition by nutrients of cell division in VBNC bacteria (33).

In this investigation, we examined the ability of an *E. coli* population exposed to HOCl stress to undergo a reversal from the VBNC state by incubation in phosphate buffer without nutrient

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addition. We show that after a few days, 10% of the initial challenged population was culturable, independent of the initial survival. We further attempted to determine whether this apparent recovery of culturability results from a resuscitation of the nonculturable population or from the development of a small culturable subpopulation by "cannibalism."

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strains used in this study were the prototroph MG1655 (1) and strain CAG12280, a *zah-281*::Tn*10* tetracycline-resistant derivative (28).

Reagents and hypochlorous acid assay. All chemicals used were analytical grade. *N*,*N*-Diethyl-*p*-phenylenediamine (DPD), propidium iodide, and sodium thiosulfate were purchased from Sigma Chemical Co.; sodium hypochlorite was purchased from Aldrich Chemical Company, Milwaukee, Wis. CTC was purchased from Polyscience. The concentration of free chlorine (hypochlorous acid plus a hypochlorite ion) was determined colorimetrically (9). Free-chlorine solutions in distilled water were stable for several hours. Under our experimental conditions (pH 7.1), approximately 66% of the sodium hypochlorite was converted to HOCl; however, we use the term HOCl to avoid confusion between free chlorine and chlorine.

Growth and hypochlorous acid challenge conditions. Cells were grown in M63 plus 0.2% glucose (18) at 25°C in a rotary shaking water bath at 200 rpm.

Hypochlorous acid challenges were carried out with cells harvested during exponential growth phase. The cells (1×10^7 to 30×10^7 bacteria/ml) were sedimented by centrifugation at $3,000 \times g$ for 10 min at 4°C, washed twice with cold 50 mM phosphate buffer (pH 7.1), and resuspended in the same volume of phosphate buffer. The number of CFU in the culture was not reduced by this washing procedure. Samples were distributed in 25-ml Erlenmeyer flasks (2.5 ml each), and hypochlorous acid (prepared daily) was added at various concentrations from 0 to 1 mg/liter (19 μM) (≤100 μl). After 20 min of incubation at 25°C in the dark with gentle shaking, free chlorine was quenched by the addition of sterile sodium thiosulfate to 500 μM. Erlenmeyer flasks used for HOCl treatment were washed with sulfochromic acid.

Cell assays and viability measurements. To determine total bacterial counts, cells were washed with phosphate buffer (50 mM, 25°C) and fixed with ethanol. They were counted by flow cytometry after being stained with propidium bromide (14). Respiring cells were monitored by the CTC reduction method as modified by Schaule et al. (26), except that cells were incubated for 2 h in M63 plus 0.2% glucose. Cells were examined at a magnification of ×1,000 with an Olympus epifluorescence microscope, employing blue light BP490 excitation and a 590-nm filter. In all cases, cells were counted in at least 10 random fields. Culturable bacteria were assayed by plating samples from suspensions onto Luria-Bertani (LB) plates after serial dilutions in phosphate buffer (50 mM, pH 7.1, 25°C). Colonies were counted after 48 h of incubation at 37°C. No increase in colony number was observed after further incubation. Dilutions were made in duplicate, and two serial dilutions were made for each sample. Values are averages from at least three counts, with variations of no more than 20% from the mean. Numbers of VBNC bacteria were estimated as the numbers of viable (respiring) bacteria minus the numbers of culturable bacteria. Numbers of dead (nonrespiring) bacteria were estimated as the total cell numbers prior to treatment minus the numbers of viable bacteria after treatment.

Resuscitation conditions. After challenge, samples of stressed and unstressed bacteria were added to fresh phosphate buffer (50 mM, pH 7.1, 25°C). Experiments were carried out in a final volume of 10 ml in 25-ml sterile glass tubes. Tubes were incubated at 25°C with gentle shaking. Samples were withdrawn for plate counts, total cell counts, and CTC counts every 24 h as described above. To ensure that no biodegradable material was released into the buffer by the glassware, the glass tubes were heated at 500°C for 2 h.

Preparation of filtrate and crude extracts. Cells were filtered onto 22-μm-pore-size filters directly or after disruption by four cycles of freezing at −80°C and thawing in a 37°C water bath.

Various calculations. Evaluation of the amount of carbon corresponding to lysed bacteria was performed according to the procedure of Neidhardt et al., the dry mass of one bacterium being evaluated at 2.8×10^{-13} g, with 50% of the dry mass being carbon (20). Estimation of the glucose equivalent of the carbon sources available from damaged bacteria was made as follows. A concentration of 10^8 cells/ml corresponds to 0.014 g of C/liter (20). Since our minimal medium, containing 0.2% glucose corresponding to 0.8 g of C/liter, sustains a plateau of 2×10^9 cells/ml, the glucose equivalent needed to reach a plateau of 10^7 can be estimated at 0.004 g of C/liter. This corresponds to 30% of the total carbon contained in 10^8 stressed cells/liter. The half-saturation constants (K_s) and maximal growth rates (μ_{max}s) for stressed and unstressed bacteria were estimated according to the weighted-regression procedure of Wilkinson (34).

RESULTS

Increase in culturable bacteria in an HOCl-stressed population in phosphate buffer, without addition of nutrients. *E.*

coli cultures harvested in exponential growth phase were stressed by incubation in phosphate buffers containing different concentrations of HOCl and then diluted 1/10 into fresh phosphate buffer. After several days of incubation, all cultures were found to contain about 5 to 10% of their initial total cell counts (Fig. 1A). As the severity of the initial stress was increased, the initial number of survivors diminished and the time required to reach the plateau phase increased. However, the rate of increase and the final number of culturable bacteria were the same regardless of the number of surviving cells after HOCl stress. No increase in culturable bacteria was observed when 100 μg of chloramphenicol per ml was added to the phosphate buffer after the stress, indicating that bacterial recovery required de novo protein synthesis (data not shown).

The percent viable cells detected by CTC ranged from 10 to 50%, depending on the HOCl treatment (Fig. 1B), and did not drop below 10% during the 12-day experiment (data not shown). The total cell count did not change significantly during the experiment and was equal to the initial number of cells (data not shown).

Untreated cells, independently of their initial concentration, did not grow in phosphate buffer: instead, they underwent a doubling (Fig. 1C), suggesting that they underwent one round of cell division.

Bacteria damaged by HOCl stress furnish nutrients for growth in phosphate buffer. A possible explanation for the observed recovery in phosphate buffer after HOCl stress was that the survivors grew by using nutrients provided by damaged bacteria.

To test whether a stressed population could release nutrients that would permit the growth of untreated cells, two *E. coli* populations which could be distinguished by genetic markers were mixed. Various concentrations (10^2 to 10^4 CFU/ml) of unstressed culturable bacteria carrying a gene conferring tetracycline resistance were mixed with a population of stressed tetracycline-sensitive bacteria. After 12 days, CFU counts on LB plates with and without tetracycline showed significant growth in the fractions of unstressed bacteria, together with recovery of the stressed cells (Fig. 2). The numbers of culturable bacteria reached by the two populations were similar, and the total number of culturable bacteria was equal to the number of culturable bacteria observed in a single initial population of stressed cells. Thus, the recovery of stressed cells could in part be explained by growth of a few culturable cells.

We tested if the level of recovery was dependent upon the amount of nutrient released by damaged bacteria. Untreated cells were incubated in phosphate buffer containing various amounts of filtered crude extract prepared from 1.5×10^8 bacteria treated with 0.8 mg of HOCl per liter. As shown in Fig. 3A, the number of CFU at the plateau increased with the concentrations of the filtered crude extract. Similar results were obtained when a direct filtrate of stressed cells was used (Fig. 3B) and when dilutions of stressed cells were incubated in phosphate buffer (Fig. 3C). At a concentration of filtrate corresponding to 4×10^5 stressed bacteria, no growth of unstressed bacteria was observed (Fig. 3A). Under these conditions, the maximum amount of nutrient provided (5×10^{-5} g of C/liter), converted into glucose equivalents, was about 1.6×10^{-5} g of C/liter, which is similar to values previously reported to be insufficient to support *E. coli* growth (27). Interestingly, with a filtrate of unstressed cells which had been resuspended in phosphate buffer for a period equal to the treatment time (20 min), weak growth of the untreated cells was observed. However, the plateau was 100-fold lower than that reached with a filtrate of an equivalent number of stressed cells (data not shown).
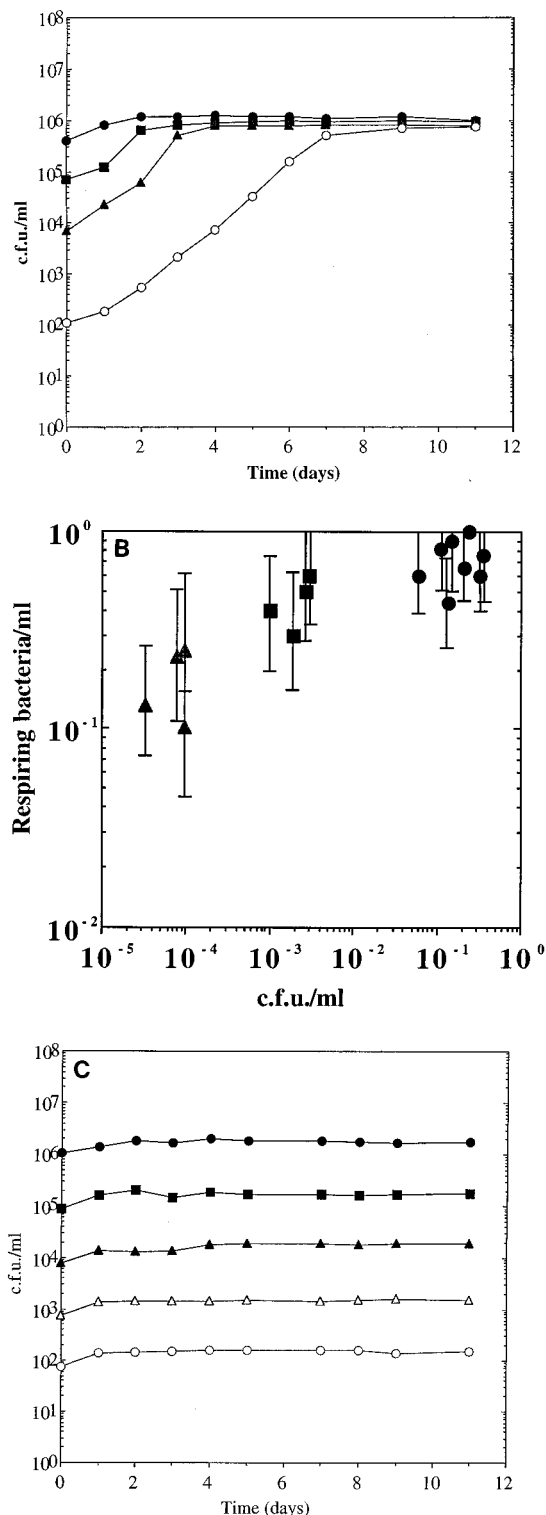
The number of CFU at the plateau reached by stressed or unstressed bacteria after incubation in phosphate buffer, plotted as a function of the number of bacteria that had been stressed (or of bacterial equivalents in the filtrate added), showed a linear relationship. This strongly suggested that culturable cells grow at the expense of damaged cells, a phenomenon referred to as cryptic growth (17).

Growth rate of recovering bacteria after HOCl stress. A comparison of the growth curves of untreated bacteria diluted in phosphate buffer supplemented with filtered crude extract of treated cells (Fig. 3A) with those of stressed bacteria diluted in phosphate buffer (Fig. 3C) revealed that the latter population had an apparently higher growth rate that became more marked as the initial cell density increased. Growth rates of stressed and unstressed cells (Fig. 4) were calculated from several experiments (such as that whose results are represented in Fig. 3) in which the total bacterial number before stress, the intensity of the stress, and the number of culturable survivors after stress were varied. The growth rate of untreated and treated bacteria typically followed the kinetics inferred by Monod (19) by analogy with Michaelis-Menten enzyme kinetics: $\mu = \mu_{max} \cdot [S/(S + K_s)]$, where μ is the growth rate and S is the nutrient concentration (Fig. 4). However, as depicted in Fig. 4B, the μ_{max} was not reached by stressed cells, even at a high cell density. Statistical analysis of the results suggested significant increases of the μ_{max}s and the K_ss in the stressed population. For the unstressed bacteria the μ_{max} (per day) was 24.5 $\pm$ 1.4 (mean $\pm$ standard deviation) and the K_s was 2.1 $\times$ $10^8 \pm 1 \times 10^8$, while for stressed bacteria the values were 4.8 $\pm$ 0.15 and $1 \times 10^7 \pm 5 \times 10^6$, respectively. Values of apparent kinetic constants were calculated according to the weighted-regression procedure of Wilkinson ($n = 12$) using the data shown in Fig. 4.

DISCUSSION

As is the case under other stress conditions, such as prolonged nutrient starvation or a shift to a low temperature, transient exposure to HOCl in nutrient-free buffer leads to a significant loss of culturability, even though a large fraction of the cells remains viable. The apparent recovery of culturability without addition of nutrients is, as shown in this study, largely due to regrowth of a few culturable cells at the expense of the damaged cells.

The level of recovery appears to be dependent on the initial stressed-cell density and is independent of the number of surviving cells after the stress. We observed that 10^8 damaged bacteria give rise to a maximum of 10^7 CFU/ml. Assuming that carbon is the growth-limiting factor, under these conditions the yield in carbon-containing medium is about 1/10 the original inoculum. This assumption was tested: addition of 0.2% glucose to the phosphate buffer allowed growth of up to 10^8 cells/ml (unpublished results). Postgate and Hunter (24) calculated that approximately 50 cells need to die to support the growth of 1 cell, a value which is consistent with those obtained here. Interestingly, filtrates of stressed cells supported the growth of untreated cells as well as the crude extracts did, suggesting that essentially all nutrients are released immedi-

FIG. 1. Increase of the culturable population of HOCl-stressed bacteria in phosphate buffer. (A) A culture of MG1655 was grown until it reached a density of 7×10^7 CFU/ml, washed and then incubated for 20 min in phosphate buffer with various concentrations of HOCl, and diluted 10-fold in phosphate buffer. Samples were withdrawn at intervals for colony counts on LB plates. HOCl concentrations were 0.4 (●), 0.6 (■), 0.8 (▲), and 1 (○) mg/liter. (B) Number of viable cells detected by the CTC method after HOCl treatment. Bacteria (10^8 CFU/ml) were treated with HOCl. Numbers expressed in CFU/milliliter are numbers of survivors counted on LB plates. Respiring cells were counted by the CTC method. Each point represents the mean of three determinations, and error bars show standard deviations. HOCl concentrations were 0.4 (●), 0.6 (■), and 0.8 (▲) mg/liter. (C) A culture was grown until it reached a density of 10^8 CFU/ml; after a wash and 20 min of incubation in phosphate buffer, it was diluted in phosphate buffer and incubated at 25°C. Samples were taken at intervals and plated for colony counts. Dilutions were 10^{-2} (●), 10^{-3} (■), 10^{-4} (▲), 10^{-5} (△), and 10^{-6} (○).
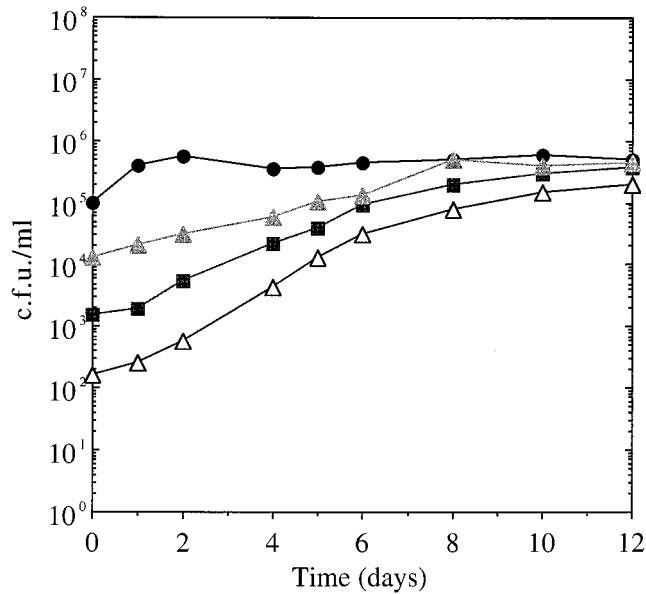
FIG. 2. Growth in phosphate buffer of untreated bacteria in the presence of HOCl-stressed bacteria. Various concentration of untreated bacteria (10^2 [△], 10^3 [■], and 10^4 [▲] CFU/ml) carrying the Tet^r marker were added to a culture containing 6×10^7 CFU/ml treated with 0.4 mg of HOCl per liter and diluted 10-fold in phosphate buffer. Samples were withdrawn at intervals for 12 days for CFU counting on LB plates (total culturable cells) and LB plates containing tetracycline (untreated bacteria). Counts of untreated bacteria (△, ■, and ▲) and values for treated bacteria (●) correspond to the mean calculated counts for the three experiments, with standard deviations of no more than 20%.

dium) but not when plated on rich LB medium is not clear. It is possible that the rich LB medium inhibits the division of VBNC cells, as suggested by Whitesides and Oliver for *V. vulnificus* (33), but the mechanism of a putative inhibition by elevated nutrient levels is not yet understood. Another possibility might relate to whether liquid or solid medium is used. Bacteria spread on plates can be considered unicellular organisms (there are large distances between the bacteria), whereas in liquid medium they may be influenced by other members of the culture, except when the medium is sufficiently diluted. For example, experiments by Ma and Eaton (16) support the concept that bacterial catalase may defend populations but not individual *E. coli* cells against environmental H_2O_2. Concentrated suspensions (5×10^7 CFU/ml) of catalase-positive *E. coli* do preferentially survive H_2O_2 challenge and can even cross-protect adjacent catalase-deficient organisms. An excess of hydrogen peroxide due to an impairment of catalase may actually play a role in the difference between solid and liquid media with regard to bacterial recovery from an HOCl stress. Indeed, the exogenous addition of catalase to various selective media increases detection of HOCl-stressed cells of *E. coli* (4). Furthermore, catalase activity is reduced in cell populations after HOCl exposure (reference 4 and our unpublished results). This impairment of catalase activity, which could be lethal for some VBNC cells, might be compensated for in liquid cultures containing high cell concentrations.

We have shown that culturable bacteria can grow at the expense of dead or damaged cells after an HOCl challenge in medium devoid of nutrients. To determine whether the few dead or damaged HOCl-treated bacteria present in drinking
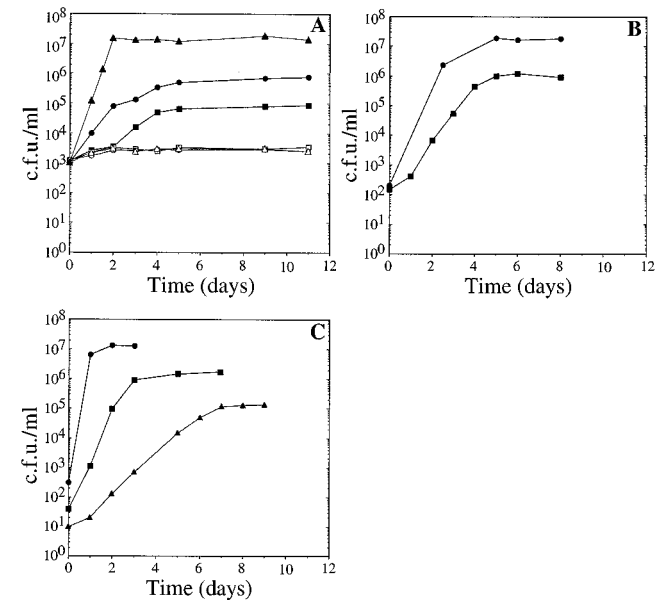
ately upon stress. However, the possibility that filtration damages the stressed cells, leading to nutrient release, cannot be excluded.

Stressed cells grew at a significantly higher rate than unstressed cells; possibly this reflected improved growth ability. In order to determine whether the population of stressed bacteria reaching the plateau had stably acquired properties conferring faster growth in stressed medium, bacteria scraped from plates were diluted in filtrate. Their growth rate was similar to that of untreated cells (unpublished results). Thus, putative stress-induced properties conferring fast growth are likely to be transient, but this makes it difficult to account for the fact that the growth rate remains high for up to 15 generations during recovery.

Alternatively, in addition to culturable cells that grow after HOCl treatment, there may be VBNC cells which recover from injuries during incubation in phosphate buffer. Stress conditions may induce response pathways that permit mildly injured VBNC cells to repair or bypass injury. Previous studies (5, 6) suggested that exposure to HOCl triggers an adaptive response(s). Recovery (resuscitation) of VBNC cells with different lag periods produces a characteristic growth pattern with an increased apparent growth rate. This was not related to a lack of nutriment, since 2×10^6 stressed cells supplemented with the filtrate of 2×10^8 stressed cells grew at the same rate as unstressed cells. This result indicates that when there are fewer than 10^7 stressed cells, the number of recoverable VBNC cells is too low to be detectable by our experimental methods. To characterize these growth patterns, a deterministic model was constructed. Preliminary simulations suggest that this phenomenon can be represented by first-order kinetics (7).

Why some VBNC cells should be able to recover in phosphate buffer supplemented with damaged bacteria (liquid me-

FIG. 3. Influence of initial cell density on growth in phosphate buffer after HOCl stress. (A) A culture was grown until it reached a density of 1.2×10^8 CFU/ml, spun down, and washed and then incubated in phosphate buffer for 20 min. Samples containing 10^3 CFU/ml were inoculated in phosphate buffer containing various dilutions of filtered crude extract obtained from 1.5×10^8 cells treated with 0.8 mg of HOCl per liter. Samples were withdrawn at intervals for cell counts. Dilutions of filtrate were none (▲), 10^{-1} (●), 10^{-2} (■), 5×10^{-3} (□), 2.6×10^{-3} (△), and 10^{-3} (○). (B) Same as panel A except that stressed cells were directly filtered, without disruption. Dilutions of filtrate were none (●) and 10^{-1} (■). (C) Cells harvested at 1.2×10^8 CFU/ml were treated with 0.8 mg of HOCl per liter and diluted in phosphate buffer. Samples were withdrawn at intervals for cell counts. Dilutions were none (●), 10^{-1} (■), and 10^{-2} (▲).
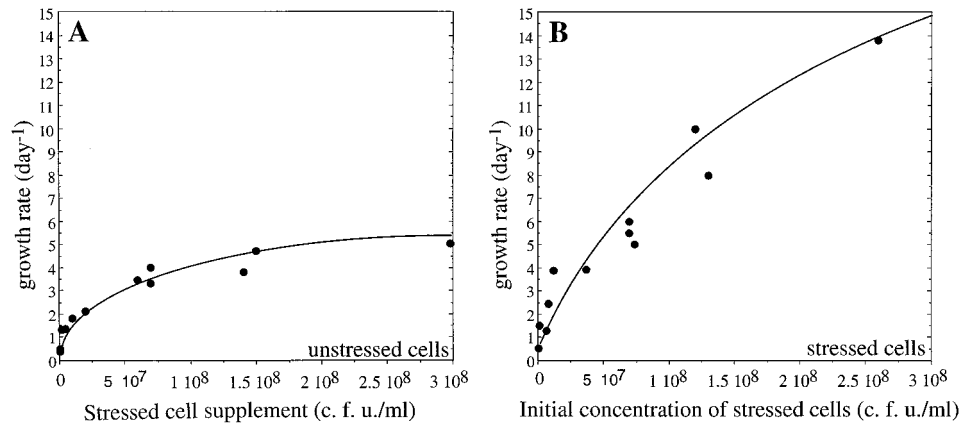
FIG. 4. Variations of growth rates of stressed and unstressed bacteria.

water could furnish enough biodegradable nutrients for growth of native microorganism populations present in an aquatic environment will need further study. In addition, the present data suggest that some HOCl-stressed *E. coli* organisms are capable of switching between the VBNC and culturable states in phosphate buffer. This recovery of culturability under particular environmental conditions constitutes a challenge for microbiologists attempting to detect these indicator organisms by currently accepted methods.

REFERENCES

1. **Bachmann, B. J.** 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 2460–2488. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Barer, M. R., L. T. Gribbon, C. R. Harwood, and C. E. Nwoguh.** 1993. The viable but non-culturable hypothesis and medical bacteriology. Rev. Med. Microbiol. **4:**183–191.
3. **Byrd, J. J., H.-S. Xu, and R. R. Colwell.** 1991. Viable but nonculturable bacteria in drinking water. Appl. Environ. Microbiol. **57:**875–878.
4. **Calabrese, J. P., and G. K. Bissonnette.** 1990. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. Appl. Environ. Microbiol. **56:**3558–3564.
5. **Dukan, S., and D. Touati.** 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. J. Bacteriol. **178:**6145–6150.
6. **Dukan, S., S. Dadon, D. R. Smulski, and S. Belkin.** 1996. Hypochlorous acid activates the heat shock and *soxRS* systems of *Escherichia coli*. Appl. Environ. Microbiol. **62:**4003–4008.
7. **Dukan, S., et al.** Unpublished data.
8. **Firth, J. R., J. P. Diaper, and C. Edwards.** 1994. Survival and viability of *Vibrio vulnificus* in seawater monitored by flow cytometry. Lett. Appl. Microbiol. **18:**268–271.
9. **Greenberg, A. E., L. S. Clesceri, and A. D. Eaton (ed.).** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
10. **Herson, D. S., B. Marthi, M. A. Payer, and K. H. Baker.** 1986. Enumeration of chlorine-stressed organisms with acridine orange 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (AOINT). Curr. Microbiol. **13:**77–80.
11. **Jiang, X., and T.-J. Chai.** 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of

viable, nonculturable cells. Appl. Environ. Microbiol. **62:**1300–1305.
12. **Kaprelyants, A. S., G. V. Mukamolova, and D. B. Kell.** 1994. Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell free spent culture medium at high dilution. FEMS Microbiol. Lett. **115:**347–352.
13. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
14. **Krishan, A.** 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. **66:**188–193.
15. **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.
16. **Ma, M., and J. W. Eaton.** 1992. Multicellular oxidant defense in unicellular organisms. Proc. Natl. Acad. Sci. USA **89:**7924–7928.
17. **Mason, C. A., G. Hamer, and J. D. Bryers.** 1986. The death and lysis of microorganisms in environmental processes. FEMS Microbiol. Rev. **39:**373–401.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Monod, J.** 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. **3:**371–394.
20. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell. A molecular approach. Sinauer Associates, Inc., Sunderland, Mass.
21. **Nilsson, L., J. D. Oliver, and S. Kjelleberg.** 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J. Bacteriol. **173:**5054–5059.
22. **Oliver, J. D., F. Hite, D. McDougald, N. L. Andon, and L. M. Simpson.** 1995. Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. Appl. Environ. Microbiol. **61:**2624–2630.
23. **Oliver, J. D.** 1995. The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol. Lett. **133:**203–208.
24. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29:**233–263.
25. **Ravel, J., I. T. Knight, C. E. Monahan, R. T. Hill, and R. Colwell.** 1995. Temperature-induced recovery of *Vibrio cholerae* from the viable but nonculturable state: growth or resuscitation? Microbiology **141:**377–383.
26. **Schaule, G., H.-C. Flemming, and H. F. Ridgway.** 1993. Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. Appl. Environ. Microbiol. **59:**3850–3857.
27. **Shehata, T. E., and A. G. Marr.** 1971. Effect of nutrient concentration on the growth of *Escherichia coli*. J. Bacteriol. **107:**210–216.
28. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
29. **Votyakova, T. V., A. S. Kaprelyants, and D. B. Kell.** 1994. Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. Appl. Environ. Microbiol. **60:**3284–3291.
30. **Wai, S. N., T. Moriya, K. Kondo, H. Misumi, and K. Amako.** 1996. Resuscitation of *Vibrio cholerae* O1 strain TSI-4 from a viable but nonculturable state by heat shock. FEMS Microbiol. Lett. **136:**187–191.

31. **Weichart, D., J. D. Oliver, and S. Kjelleberg.** 1992. Low temperature induced non-culturability and killing of *Vibrio vulnificus*. FEMS Microbiol. Lett. **100:** 205–210.

32. **Weichart, D., and S. Kjelleberg.** 1996. Stress resistance and recovery potential of culturable and viable but nonculturable cells of *Vibrio vulnificus*. Microbiology **142:**845–853.

33. **Whitesides, M. D., and J. D. Oliver.** 1997. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl. Environ. Microbiol. **63:**1002–1005.

34. **Wilkinson, G. N.** 1961. Statistical estimations in enzyme kinetics. Biochem. J. **80:**324–332.

35. **Zimmermann, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36:**926–935.