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Formation of Nonculturable *Vibrio vulnificus* Cells and Its Relationship to the Starvation State

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Entry into the viable but nonculturable state by the human bacterial pathogen *Vibrio vulnificus* in artificial seawater microcosms was studied. In contrast to the long-term culturability exhibited by cells incubated under these starvation conditions at room temperature, cells exposed to a temperature downshift to 5°C exhibited an immediate decrease in culturability. Cells incubated at low temperature exhibited a morphological change from rods to cocci but demonstrated no reductive division. Of 10 factors studied which might affect the nonculturable response in *V. vulnificus*, only the physiological age of the cells was found to significantly affect the rate at which cells became nonculturable. The nonculturable response appears to be related to the starvation response, as prestarvation at room temperature for 24 h was found to eliminate the nonculturable response of cells subsequently incubated at 5°C. This observation suggests that the synthesis of starvation proteins may repress the viable but nonculturable program displayed during low-temperature incubation. The possible ecological significance of these findings is discussed.

Vibrio vulnificus is an estuarine bacterium capable of causing rapidly fatal infections in humans by producing a primary septicemia following ingestion, typically of raw oysters, or a rapidly spreading cellulitis following entry from seawater into a wound (for a recent review, see reference 27). Although isolated especially from the warmer waters of the southeastern and Gulf coasts (12, 29, 30), *V. vulnificus* has also been reported from both the northern East Coast (31) and northern West Coast (11) of the United States during the warmer months. The general inability to isolate *V. vulnificus* or other estuarine vibrios (4, 5) during the winter months or from colder waters has been suggested to result from the sensitivity of these bacteria to colder temperatures (27). Studies with *V. vulnificus* (15, 40) have shown, however, that this sensitivity results not in cell death but in entry into what has been termed the "viable but nonculturable" state (35). Cells in such a state can be shown to be viable by using several methods, e.g., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride reduction (which indicates individual cell viability through the demonstration of an active electron transport chain), but they are no longer culturable in or on media which normally support the growth of the organism. Such a state has now been demonstrated for *Escherichia coli* (3, 41), *Salmonella enteritidis* (36), *Shigella* species (6), *Aeromonas salmonicida* (1), *Campylobacter jejuni* (34), and *Vibrio cholerae* (6, 41).

In the case of *V. vulnificus*, the nonculturable state has been shown to be highly temperature dependent, with entry occurring at 5°C but not at 10°C or higher (40). Entry of *V. vulnificus* into the nonculturable state occurs at 5°C even at elevated nutrient concentrations (28), suggesting a process possibly distinct from the starvation response reported for other marine vibrios (13, 22, 23, 25). As with the starvation response, however, nonculturable cells of *V. vulnificus* exhibit marked membrane fatty acid and ultrastructural modifications compared with their culturable counterparts (15). Such cells also demonstrate a greatly reduced amino acid transport (28).

Despite the increasing recognition of a nonculturable state among a variety of bacteria and its potential importance to public health microbiology (28) and to the detection of environmentally released microorganisms (6), as well as to general microbial ecology, little is known regarding the parameters which may play a role in the entry of bacteria into this state. Here we report on a variety of factors affecting this process in *V. vulnificus* and on the interplay of the starvation response and entry into the nonculturable state.

MATERIALS AND METHODS

Growth conditions and induction of nonculturability. A nonencapsulated (translucent) (37) isolate of *V. vulnificus* C7184 was employed in these studies. Cells were typically grown in MSWYE broth (0.1% yeast extract and 0.1% Difco Proteose Peptone per liter of a "three-salts" diluent containing 0.4 M NaCl, 0.028 M $MgSO_4 \cdot 7H_2O$, and 0.01 M KCl) at room temperature (ca. 25°C) with shaking. In some studies, the defined growth medium M9 (16) with 1% glucose as the carbon source or VNSS (18) was employed. The standard method for inducing the nonculturable state was to directly inoculate flasks containing an artificial seawater (ASW) solution (17) with MSWYE-grown cells to give a final concentration of 1% of the inoculum cell count. The microcosms, initially at room temperature, were then placed in a 5°C cold room and maintained at that temperature in a static state. The time required for the microcosm temperature to decrease to 5 to 7°C was typically 2 h. For some experiments, identically prepared cells were maintained at room temperature. A series of studies was also undertaken to examine a variety of factors which might influence the rate of nonculturability of *V. vulnificus*; these are discussed in Results.

Cell and viability enumeration. Cell culturability was determined by using MSWYE agar or LB15 agar (16), with both dilution tubes and plates initially at 5°C. Plates were

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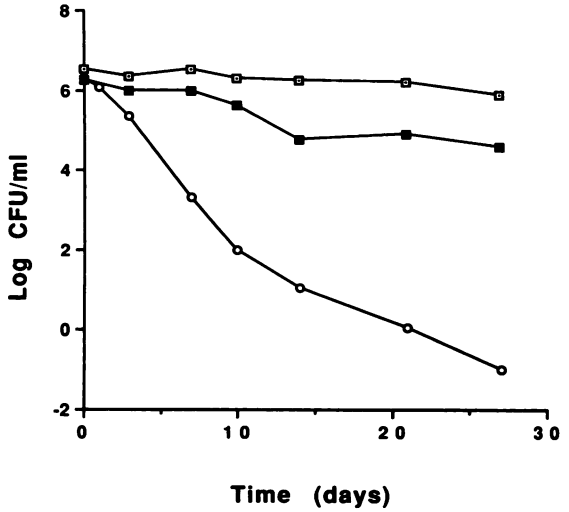

FIG. 1. The viable but nonculturable response of stationary-phase cells of *V. vulnificus*. Plate counts (○) on LB agar, direct viable (INT-positive) counts (■), and acridine orange direct counts (□) were conducted for cells grown in VNSS broth at room temperature and inoculated in an ASW microcosm held at 5°C.

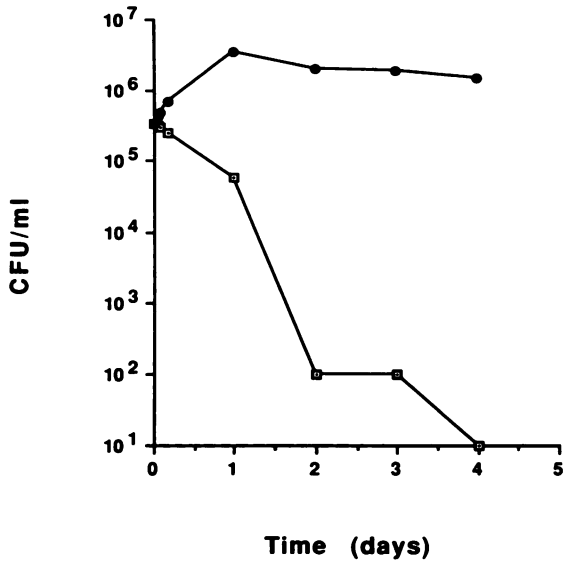

FIG. 2. Comparison of the starvation (room temperature) and nonculturable (5°C) response of logarithmic-phase cells of *V. vulnificus*. Plate counts (LB agar) were made on cells grown to logarithmic phase in M9 broth plus glucose at room temperature (OD_{610}, 0.09) and incubated in ASW+N+P microcosms held at 5°C (□) or room temperature (●).

subsequently incubated at 37°C. Cells were considered to be nonculturable when a typical concentration of <1 CFU/ml of microcosm was attained. Respiring cells were monitored by the INT reduction method as modified by Oliver and Wanucha (28), and total cell number was determined by the acridine orange method (39). Cells were examined at a magnification of ×1,250 with an Olympus BH-2 epifluorescence microscope, employing blue light (390 to 490 nm) excitation and a 515-nm filter. In all cases, cells in at least 10 random fields were counted. Cell sizes were calculated with an ocular micrometer.

Effect of prestarvation on entry into the nonculturable state. Cells were grown to the logarithmic phase (optical density at 610 nm [OD_{610}], 0.135) in MSWYE at room temperature and inoculated into 250 ml of ASW. These microcosms were incubated at room temperature for various periods of up to 48 h, at which time 25-ml aliquots were cooled to 5°C. Plate counts were performed after the cells had been at 5°C for 24 h and thereafter at approximately daily intervals. Plate counts were also performed on control cells which were maintained at room temperature throughout the study.

RESULTS

When cells of *V. vulnificus* were shifted to 5°C, plate counts declined gradually, typically from ca. 10^6/ml to less than 10^{-1} CFU/ml (Fig. 1). Throughout the entire process of entry into this nonculturable state, no changes in total cell counts were observed, and direct viable counts (as measured by INT-reducing cells) declined only to ca. 10^5 over the 30-day period (Fig. 1). Cells exposed to the temperature downshift also underwent a morphological change from rods to predominantly cocci (diameter, 0.8 to 1.0 μm) but did not appear to undergo the reductive division seen during starvation in room temperature microcosms (see below). Cells subjected to the temperature downshift did not appear to be committed to nonculturability, however. In one study, when cells which were incubated for 7 days at 5°C (at which time the population had decreased from an initial 10^6 CFU/ml to 1.6×10^2 CFU/ml) were transferred to room temperature, the culturable population density exceeded 10^3 after only 4 h.

In contrast to the decline observed when cells were incubated at 5°C, the same cell population inoculated into microcosms maintained at room temperature responded to the multiple starvation conditions with a process of reductive division (Fig. 2). Such division, which was detectable from the earliest (1-h) sampling, reached a maximum within 24 h, at which time cell populations as much as a full log higher than the original inoculum were generally obtained. The same increase in culturable cell numbers occurred whether the microcosms were inoculated directly from growth cultures or whether contaminating nutrients were removed by centrifugation (as in Fig. 2). Cells resulting from this reductive division process changed from rods, originally 1.7 by 0.8 μm to 2.9 by 0.8 μm for logarithmic-phase cells at the time of inoculation, to a mixture of cocci ca. 0.5 to 0.65 μm in diameter and rods 1.4 by 0.6 μm to 1.7 by 0.8 μm by day 15 of the starvation response. Such starved populations typically maintained culturable cell densities of 10^4 to 10^5 CFU/ml after 90 days of incubation at room temperature. In contrast, cells shifted to 5°C again exhibited an immediate decrease in culturability (Fig. 2).

A series of studies were undertaken in an attempt to clarify what environmental or manipulation parameters might be responsible for the variations in time to nonculturability often observed, as typified in Fig. 1 and 2. Figure 3, which shows the entry into the nonculturable state by cells grown at either room temperature or 37°C and the effect of microcosm incubation temperature, illustrates our general observation that induction of the nonculturable response is dependent on the microcosm incubation temperature but does not appear to be markedly affected by cell growth temperature. Similarly, no major effects on the rate of nonculturable development were observed (data not shown)

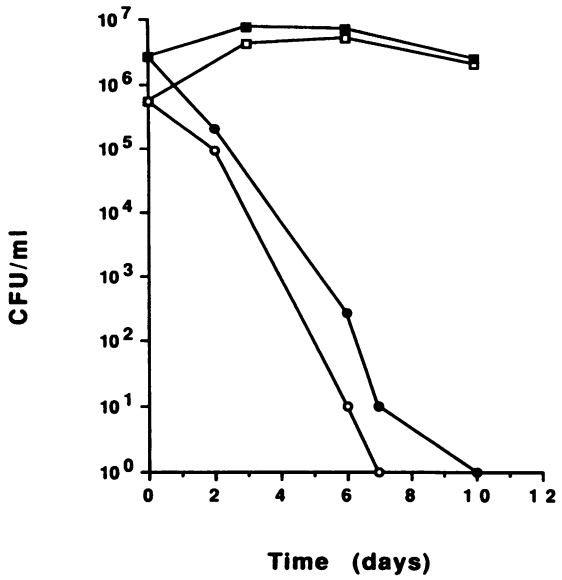

FIG. 3. Effect of growth temperature and microcosm incubation temperature on development of the nonculturable response in *V. vulnificus* C7184. Cells were cultured in MSWYE broth at room temperature or 37°C to early- to mid-logarithmic phase and inoculated into ASW microcosms held at 5°C or room temperature, and plate counts were performed. Symbols indicate cells grown at room temperature and transferred to room temperature (□) or 5°C (○) and cells grown at 37°C and transferred to room temperature (■) or 5°C (●).

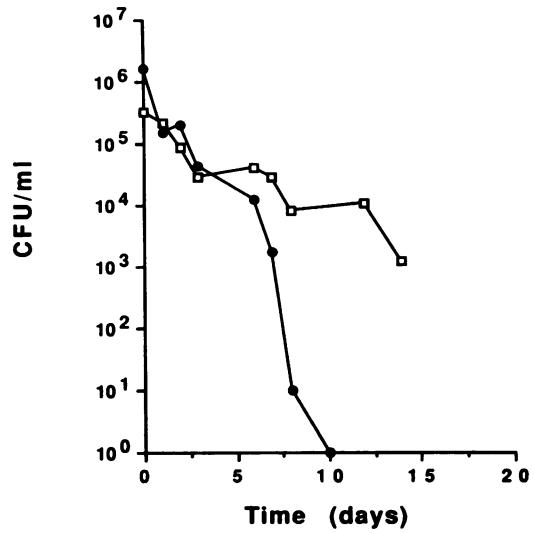

FIG. 4. Effect of growth phase on entry into the nonculturable state. Cells of *V. vulnificus* C7184 were cultured in M9 broth plus glucose at room temperature to logarithmic phase (OD_{610}, 0.15) (●) or stationary phase (OD_{610}, 0.95) (□). Cells were inoculated into ASW+N+P microcosms held at 5°C, and samples were taken periodically for plateable cells.

when a different strain (CVD713t) was employed, when the ASW microcosm diluent was modified to include a nitrogen and phosphate source (to examine carbon starvation versus multiple starvation for carbon, nitrogen, and phosphate), whether cells were washed or not prior to microcosm inoculation, or regardless of the initial cell density (6×10^5 to 4×10^7 CFU/ml), the initial plate temperature (5 or 25°C), or the plate incubation temperature (15, 25, or 37°C). The chill rates of microcosms of identical volume (100 ml) contained in 125- to 1,000-ml flasks did not differ significantly, and no effect on time to nonculturability was observed. The salt content of the plating medium was varied to include 0.5, 1, 2, or 3% NaCl; only at 3% did salt appear to have some negative effect on the culturability of the cells.

In contrast, a significant difference in time to nonculturability between cells taken from the logarithmic growth phase and cells taken from the stationary phase was observed. As seen in Fig. 4, cells taken from stationary phase (OD_{610}, 0.95) generally required about twice as many days or more to become nonculturable when exposed to a temperature downshift from 25 to 5°C than did logarithmic (OD_{610}, 0.15) cells. Indeed, when the optical density of the starting inoculum was plotted against the slope of the time course of nonculturability (Fig. 5), an excellent correlation (r = 0.9988) was observed.

The observation that stationary-phase cells required a longer time to enter the nonculturable state than log-phase cells suggested that nutrient starvation, or some other stationary-phase-induced stress response, might play a role in the nonculturable process. To examine this possibility, cells were incubated under carbon starvation conditions at room temperature for various times prior to temperature downshift. The results of this study are seen in Fig. 6. As is evident, cells placed at 5°C coincident with exposure to starvation conditions were observed to undergo an immediate decline in culturability. Whereas cells prestarved for 1 to 2 h at room temperature initiated the expected starvation-induced reductive division, they exhibited an immediate decline in culturability on temperature downshift. Prestarvation at room temperature for as little as 4 h, however,

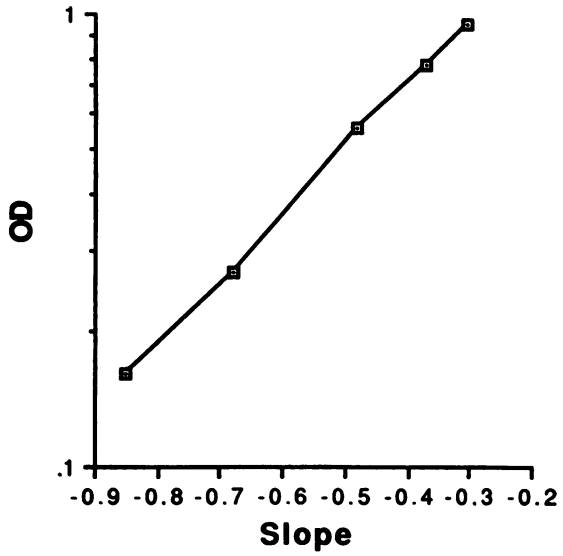

FIG. 5. Correlation of initial OD_{610} and slope calculated for the plate counts of cells incubated in 5°C microcosms. Cells of *V. vulnificus* C7184 were inoculated into microcosms following growth in MSWYE broth to OD_{610}s ranging from 0.135 to 1.00. Slopes of the nonculturable entry curves were calculated by using a linear curve fit of the logarithmic values. The regression coefficient (0.9988) was determined for the resulting curve shown in the figure.

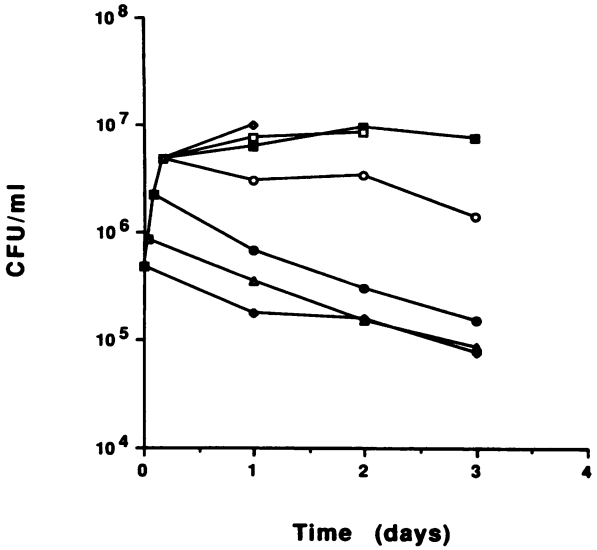

FIG. 6. Effect of prestarvation of *V. vulnificus* C7184 on entry into the nonculturable state. Cells were cultured to mid-logarithmic phase (OD_{610}, 0.135) in MSWYE broth at room temperature and diluted into an ASW microcosm held at room temperature. At intervals, 25-ml aliquots were cooled to 5°C, and plate counts were determined at daily intervals. Cells were preincubated at room temperature for 0 (◆), 1 (△), 2 (●), 4 (○), 24 (□), or 48 (◇) h prior to transfer to 5°C. Also shown is the control (■) of cells maintained constantly at room temperature.

resulted in a decrease in the rate of this decline, and cells prestarved for 24 h or more exhibited no decrease in culturability when incubated at 5°C. Cells placed immediately at 5°C, and those prestarved for less than 4 h, reached nonculturability within 13 days, whereas cells starved for longer periods at room temperature maintained a cell density of at least 10^5 CFU/ml throughout the study.

DISCUSSION

V. vulnificus enters into the viable but nonculturable state when exposed to temperatures below 10°C (Fig. 1) (40). The nonculturable response is quite different from the well-described starvation response of marine bacteria (21–23, 25, 26) which we observed when cells of *V. vulnificus* were incubated in microcosms identical to those used for the nonculturable response, but at 25°C instead of 5°C (Fig. 2). While these cells were observed to undergo a size reduction when exposed to the temperature downshift, they do not respond with an increase in cell number (Fig. 1 to 4 and 6). This response is the same as that described in our earlier studies of this bacterium (15, 28). The cells appeared to respond quickly to the temperature downshift, with an immediate decrease in culturability observed by the first (1-h) sample, whereas the time required to reach 5 to 7°C was ca. 2 h.

In the experiments conducted for the present study, cells of *V. vulnificus* were observed to become nonculturable in less than 4 to over 24 days, depending on the conditions employed. Similar variations in the time for nonculturability for different strains of the same bacterial species (3, 40, 41), and even of the same strains (8, 15, 28, 40), have been reported. Few of these studies have reported on those factors, other than microcosm incubation temperature, which might affect the rate of the nonculturable response. Aeration (34), salinity (8), and cell washing (8, 41) have been reported to affect culturability. Our results clearly demonstrate that, of the many parameters we examined, the physiological age of the cells inoculated into the microcosms was the only factor to have a major effect on the rate at which *V. vulnificus* entered the nonculturable state. That bacteria in the logarithmic phase of growth are more sensitive to cold, as well as to a variety of other stresses, than are cells in the stationary phase has been long recognized. However, that the apparent sensitivity to cold may result not in cell death but in the viable but nonculturable response has only more recently been recognized. In the case of *V. vulnificus*, the formation of nonculturable cells from stationary-phase cultures was significantly delayed compared with cells taken from early- to mid-logarithmic-phase cultures (Fig. 4). A comparison of the data presented in Fig. 4 and 6, however, reveals that the stationary phase allows for only partial repression of the viable but nonculturable response, whereas increased starvation, e.g., for 24 h or longer, completely inhibits this response. This raises interesting questions concerning the role of stationary-phase- or starvation-specific gene expression. The induction of genes specific for these stages in the bacterial life cycle is well documented (14, 19, 20, 26, 38). The coordinated response to stationary phase, or to carbon or multiple starvation, has been reported to involve the induction of stimulons and regulons, some of which overlap with other known stress responses such as the heat shock regulon (19, 25, 26). It appears that the nutrient starvation conditions employed in the present study induce such responses in *V. vulnificus* and that these may repress the viable but nonculturable program displayed during the low-temperature incubation. However, whereas a temperature downshift has been shown to quickly and significantly reduce the rate of protein synthesis in *E. coli* (2), it has been shown to induce or to greatly enhance the synthesis of 13 proteins in this bacterium (7, 10). Thus, the conditions employed in the present study might induce not only starvation proteins in *V. vulnificus* but cold shock proteins as well. The interactions of these two stresses (nutrient starvation and temperature downshift) is likely to be complex and is currently being investigated in our laboratories.

In their classic study on the survival of starved bacteria, Postgate and Hunter (33) reported on a variety of parameters which affect the survival of cells undergoing nutrient starvation. In that study, the term "dead" was used to describe ". . . bacteria that failed to multiply in the arbitrary favourable environment provided. . ." Such a definition has been widely held by microbiologists, who are primarily concerned with populations as opposed to single bacterial cells. However, as later realized by Postgate (32), bacteria may lose their ability to form colonies and yet remain functional as individual cells. Indeed, Hoppe (9) has suggested that nonculturable cells are primarily responsible for organic turnover in the open ocean. The possibility of resuscitating such cells could provide a mechanism whereby nonculturable cells, in a metabolically dormant condition, could revert to a state of increased metabolism when environmental conditions are more conducive. Indeed, we have recently reported that the complete resuscitation of nonculturable cells of *V. vulnificus* is possible, without the addition of exogenous nutrient, simply by providing the cells with a temperature upshift for 2 to 3 days (24).

Whether or not the nonculturable state presents a survival advantage for cells exposed to cold temperatures in the natural environment has yet to be demonstrated. Because the resuscitation of nonculturable cells is possible, however,

it seems likely that this state represents a second survival mechanism along with the starvation response. Indeed, the two responses appear to be linked, with starvation preventing the nonculturable response. Such inhibition is likely due to the synthesis of starvation-induced stress proteins, which are known to be cross protective for a variety of other stresses (19, 20).

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REFERENCES

1. **Allen-Austin, D., B. Austin, and R. R. Colwell.** 1984. Survival of *Aeromonas salmonicida* in river water. FEMS Microbiol. Lett. **21**:143–146.
2. **Broeze, R. J., C. J. Solomon, and D. H. Pope.** 1978. Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. J. Bacteriol. **134**:861–874.
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. **Chowdhury, M. A. R., H. Yamanaka, S. Miyoshi, K. M. S. Aziz, and S. Shinoda.** 1989. Ecology of *Vibrio mimicus* in aquatic environments. Appl. Environ. Microbiol. **55**:2073–2078.
5. **Chowdhury, M. A. R., H. Yamanaka, S.-I. Miyoshi, and S. Shinoda.** 1990. Ecology and seasonal distribution of *Vibrio parahaemolyticus* in aquatic environments of a temperate region. FEMS Microbiol. Ecol. **74**:1–10.
6. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3**:817–820.
7. **Goldstein, J., N. S. Pollitt, and M. Inouye.** 1990. Major cold shock protein of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87**:283–287.
8. **Hoff, K. A.** 1989. Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. Appl. Environ. Microbiol. **55**:1775–1786.
9. **Hoppe, H.-G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of autoradiography. Mar. Biol. **36**:291–302.
10. **Jones, P. G., R. A. vanBogelen, and F. C. Neidhardt.** 1987. Induction of proteins in response to low temperature in *Escherichia coli*. J. Bacteriol. **169**:2092–2095.
11. **Kaysner, C. A., C. A. Abeyta, M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. Appl. Environ. Microbiol. **53**:1349–1351.
12. **Kelly, M. T.** 1982. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf Coast environment. Appl. Environ. Microbiol. **44**:820–824.
13. **Kjelleberg, S., M. Hermansson, P. Mårdén, and G. W. Jones.** 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Annu. Rev. Microbiol. **41**:25–49.
14. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary phase gene expression in *Escherichia coli*. Mol. Microbiol. **5**:49–59.
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. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Mårdén, P., M. Hermansson, and S. Kjelleberg.** 1986. Incorporation of tritiated thymidine by marine bacterial isolates when undergoing a starvation survival response. Arch. Microbiol. **149**:427–432.
18. **Mårdén, P., A. Tunlid, K. Malmcrona-Freiberg, G. Odham, and S. Kjelleberg.** 1985. Physiological and morphological changes during short term starvation of marine bacterial isolates. Arch. Microbiol. **142**:326–332.
19. **Matin, A.** 1990. Molecular analysis of the starvation stress in *Escherichia coli*. FEMS Microbiol. Ecol. **74**:185–195.
20. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Annu. Rev. Microbiol. **43**:293–316.
21. **Morita, R. Y.** 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. **6**:171–198.
22. **Morita, R. Y.** 1985. Starvation and miniaturisation of heterotrophs, with special emphasis on maintenance of the starved viable state, p. 111–130. *In* M. Fletcher and G. Floodgate (ed.), Bacteria in their natural environments: the effect of nutrient conditions. Academic Press, Inc., New York.
23. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34**:436–441.
24. **Nilsson, L., J. D. Oliver, and S. Kjelleberg.** 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J. Bacteriol. **173**:5054–5059.
25. **Nyström, T., N. H. Albertson, K. Flärdh, and S. Kjelleberg.** 1990. Physiological adaptation to starvation and recovery from starvation by the marine *Vibrio* S14. FEMS Microbiol. Ecol. **74**:129–140.
26. **Nyström, T., K. Flärdh, and S. Kjelleberg.** 1990. Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **172**:7085–7097.
27. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 569–599. *In* M. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York.
28. **Oliver, J. D., and D. Wanucha.** 1989. Survival of *Vibrio vulnificus* at reduced temperatures and elevated nutrient. J. Food Saf. **10**:79–86.
29. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1982. Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. Appl. Environ. Microbiol. **44**:1404–1414.
30. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45**:985–998.
31. **O'Neill, K. R., S. H. Jones, and D. J. Grimes.** 1990. Incidence of *Vibrio vulnificus* in northern New England water and shellfish. FEMS Microbiol. Lett. **72**:163–168.
32. **Postgate, J. R.** 1976. Death in macrobes and microbes. Symp. Soc. Gen. Microbiol. **26**:1–18.
33. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–263.
34. **Rollins, D. M., and R. R. Colwell.** 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. **52**:531–538.
35. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies in the natural environment. Microbiol. Rev. **51**:365–379.
36. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but not recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30**:334–337.
37. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect. Immun. **55**:269–272.
38. **Tormo, A., M. Admiron, and R. Kolter.** 1990. *surA*, an *Escherichia coli* gene essential for survival in stationary phase. J. Bacteriol. **172**:4339–4347.
39. **Van Es, F. B., and L.-A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria. Adv. Microb. Ecol. **6**:111–170.
40. **Wolf, P. W., and J. D. Oliver.** Submitted for publication.
41. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Atwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8**:313–323.