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Effect of Low Temperature on Starvation-Survival of the Eel Pathogen *Vibrio vulnificus* Biotype 2

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At present, no reports exist on the isolation of the eel pathogen *Vibrio vulnificus* biotype 2 from water samples. Nevertheless, it has recently been demonstrated that this biotype can use water as a route of infection. In the present study, the survival of this pathogen in artificial seawater (ASW) microcosms at different temperatures (25 and 5°C) was investigated during a 50-day period, with biotype 1 as a control. *V. vulnificus* biotype 2 was able to survive in the culturable state in ASW at 25°C in the free-living form, at least for 50 days, entering into the nonculturable state when exposed to low temperature. In this state, this microorganism survived with reduced rates of activity, showing marked changes in size and morphology. The rate at which cells became nonculturable was dependent on their physiological age. The capsule seems not to be necessary for the survival of biotype 2 in aquatic environments as a free-living organism. Culturability remained the highest on modified salt water yeast extract agar, which is closer in salt and nutrient composition to ASW than heart infusion agar. Biotype 2 cells recovered culturability on solid media after an increase of incubation temperature from 5 to 25°C. Culturable cells of this bacterium maintained infectivity for either eel or mice, while dormant cells seemed to lose their virulence. The former finding suggests that the aquatic environment is a reservoir and vehicle of transmission of this pathogen.

Vibrio vulnificus is an estuarine bacterium which comprises two biotypes. Biotype 1 is an opportunistic human pathogen, ubiquitous in the marine environment, capable of causing either primary septicemia after ingestion of raw shellfish or water-borne infections after exposure to seawater (22). Biotype 2 is classically considered an obligate eel pathogen since until now it has been isolated only from diseased eels and no reports exist on its occurrence in water samples. This biotype seemed to be restricted to Japan until it was recently recovered from diseased eels in Taiwan (31) and Spain (8). From November 1989 to July 1990, biotype 2 produced several epizootic outbreaks with variable mortality rates in a brackish-water Spanish eel farm, until the water salinity decreased to 3‰ (7, 8, 11). During this almost 1-year period this microorganism was never recovered from the water of the culture tanks, while biotype 1 was isolated from either the surface of healthy eels or tank water (7, 11). Despite a decrease in water salinity, three outbreaks were recorded in widely separated periods (1990, 1992, and 1994), coinciding with higher temperatures (2, 7, 11).

V. vulnificus biotype 2 grows rapidly in the rich medium represented by the eel (10) and may adopt a starvation-survival state when released into the nutrient-diluted environment of culture tanks. We have recently demonstrated, for the first time, that biotype 2 is able to infect eels by the water-borne route, with transmission dependent on the presence of the capsule, as well as temperature and water salinity (4). Therefore, this pathogen must survive as well in water when using it as a route of infection. However, no attempt has been made to determine if this bacterium is able to survive in aquatic environments, away from its natural host, and if so, whether it becomes nonculturable. The former would be one of the reasons for the lack of reports of biotype 2 from water samples. On the contrary, there is a considerable amount of data concerning the starvation-survival mechanisms displayed by *V. vulnificus* biotype 1 (for a review, see reference 23) in both artificial seawater (20, 27, 37) and natural seawater (26) related to the formation of the nonculturable state and its relationship to human pathogenicity (17, 24). From the overall data some points could be highlighted. (i) Cells of biotype 1 exhibit a long-term culturability in nutrient-limited artificial seawater microcosms at room temperature but become nonculturable at low temperature (27). This dormant state is defined as an inability of bacterial cells to produce colonies on appropriate solid media even following prolonged incubation under suitable conditions (12, 17, 29, 39). (ii) The rate at which cells become nonculturable is significantly affected by the physiological age of the cells (27). (iii) Induction of nonculturability is independent of the presence or absence of a polysaccharide capsule that confers pathogenicity for mice (30). (iv) Nonculturable cells can be resuscitated to the plateable state just by an increase in temperature, without the addition of a nutrient (20, 26). This suggests that temperature may be the determining factor in either resuscitation or formation of nonculturable cells in estuarine environments (20, 26, 36). (v) Dormant cells of biotype 1 retain virulence for laboratory animals (24). As a consequence, this biotype represents a potential public health hazard for water-borne infections, even in a dormant state.

The ability of pathogens to survive and remain infective in the external environment is considered a major factor in the epidemiology of diseases. In this study, we have focused our investigations on clarifying whether *V. vulnificus* biotype 2 is capable of free-living existence under the nutrient starvation conditions usually found in aquatic environments and if it becomes nonculturable on solid laboratory media. The influences of low temperature, the cells' physiological state, and presence of the capsule on induction of the nonculturable state were also investigated. Infectivity was evaluated by using eels and mice as experimental animals.

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MATERIALS AND METHODS

Bacterial strains and microcosm conditions. Two strains of *V. vulnificus* with different origins and sources of isolation were used in this study: biotype 2 strain E22 was recovered from internal organs of a diseased European eel in Spain (8), and the biotype 1 clinical isolate, C7184, was used as control. Strains were maintained as lyophilized stocks at room temperature. Cells were grown to stationary phase in modified salt water yeast extract (MSWYE) broth (25) at room temperature (25°C) with shaking. Growth of cells used as the inoculum was measured either at optical density at 610 nm or as CFU per milliliter of culture on MSWYE agar. For starvation experiments, MSWYE-grown cells were inoculated into flasks containing a sterilized artificial seawater (ASW) solution (18) to give a final concentration of 1% of the inoculum cell count. Microcosm incubations were made in the static state. For inducing the nonculturable state, flasks with identically prepared cells were placed at 5°C and mantained at this temperature throughout the experiment, as described by Linder and Oliver (17). A series of studies was also undertaken to examine some factors which may influence the rate of nonculturability of *V. vulnificus* biotype 2 at reduced temperature. These were the effects of prestarvation of the cells, the cells' physiological state, and the presence of capsule on induction of the nonculturable state. To determine the effect of prestarvation, ASW microcosms were incubated for 48 h at room temperature before cold incubation at 5°C. The influences of physiological state and capsular material were determined by inoculating opaque (encapsulated) and translucent (nonencapsulated) cells from both logarithmic and stationary growth phases into ASW microcosms and chilling at 5°C. During incubation of the microcosms at 25 and 5°C, cells were observed microscopically, and changes in morphology and size were monitored.

Cell and viability enumeration. Time-zero (inoculation time) and subsequent samples were taken for plate counts, total direct cell counts, and direct viable counts. Cell culturability was determined by aseptically removing samples from each flask and diluting in ASW. To determine any possible effect of the nutrient and salt composition of the solid media employed in the plate counts, two media were used: MSWYE agar and heart infusion (HI) agar. From 10-fold dilutions, 10-μl aliquots were plated in triplicate on both MSWYE and HI plates by the drop plate method (15). The plates were incubated at room temperature until the number of colonies present was counted (from 24 h to a week). To determine whether culturable cells persisted by the end of the incubation period, samples of 1 ml taken directly from the microcosms were seeded onto MSWYE and HI plates and observed for growth.

Total cell counts were monitored by acridine orange epifluorescence as described by Oliver (21). Metabolic activity was determined by acridine orange direct viable counts by using a modification of the antibiotic concentration and incubation time used in the direct count method of Kogure et al. (16). Briefly, samples were enriched to a final concentration of 0.25% yeast extract (Difco) in the presence of 0.05% nalidixic acid (Sigma) instead of 0.02% and incubated at room temperature for 8 h instead of 6 h. Following fixation with formalin and staining with a 0.1% acridine orange solution (21), a known volume of cells was filtered onto an irgalan black-stained, 0.2-μm-pore-size Nuclepore polycarbonate filter. Cells were examined at a magnification of ×1,250 with an Olympus BH-2 epifluorescence microscope, employing blue light excitation and a 515-nm filter. In all cases, cells in at least 20 random fields were counted. This method works by inducing growth in response to the yeast extract, whereas the antibiotic prevents cell division by inhibiting DNA synthesis. The procedure results in easily detectable, elongated cells which are considered to be viable. By counting the total number of cells and the number of elongated cells, both the total cell number and the number of metabolically active cells can be determined.

Resuscitation conditions. When culturable cells were below the detection limit (one cell per ml) by plate counts, resuscitation of the nonculturable cells was attempted by placing the flasks at room temperature in a static state. Samples were processed for enumeration by plate counts, total direct cell counts, and direct viable counts every 24 h, as described above. During resuscitation, cells were also observed microscopically.

Virulence assays. Assays for pathogenicity were made on elvers (juvenile European eels) (10 g average) and mice (20 g average) by intraperitoneal inoculation (5, 10). The 50% lethal dose (LD_{50}) was calculated by the Reed and Münch method (28) for biotype 2 cells before stress, under starvation conditions, and in the state of nonculturability (including dead cells). Because the virulence of both biotypes of *V. vulnificus* has been shown to be markedly increased when the levels of iron in serum are elevated (5, 7, 38), nonculturable cells were inoculated in phosphate-buffered saline (PBS) 2 h (for mice) or 24 h (for eels) after iron pretreatment with 0.1 ml (250 μg) of Desferal (a hydroxamate-type siderophore, which clearly increases the susceptibility of mice and eels to the infection produced by biotype 2 cells [5, 7]). Mortalities were recorded daily during a 7-day period.

RESULTS

Survival curves. The effect of starvation on culturability of *V. vulnificus* biotype 2 cells after 50 days at 25°C is shown in Fig. 1. In general, during the first 2 days, the number of culturable cells increased slightly and then gradually decreased to cell densities between 10^5 to 10^6 CFU/ml over the experimental period. At the onset of the experiment, initial increases in both total and viable cell counts were observed by epifluorescence microscopy. Subsequently, the total counts remained nearly constant at cell densities of about 10^7 bacteria per ml, whereas the viable counts declined by <1 logarithmic unit during a 50-day period (Fig. 1). When biotype 2 cells were maintained at 5°C, plate counts declined gradually, from 10^7 CFU/ml to less than 10^1 CFU/ml, in 35 days because of a gradual increase of the incapability of the cells to grow on solid media (Fig. 2). Throughout the process of entry into this nonculturable state, no significant changes in total cell counts were observed and direct viable counts declined only around 1.6 logarithmic units during the 35-day period (Fig. 2). Cells exposed to the temperature downshift also underwent morphological changes paralleling the changes in their culturability, with cells changing from rods to smaller forms with a coccoid appearance. The biotype 1 strain used as control showed a

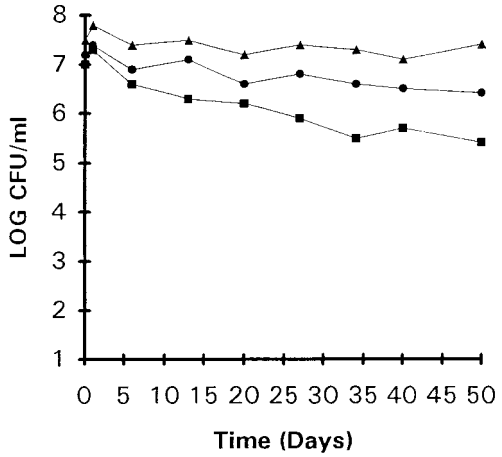

FIG. 1. Starvation-survival response of stationary-phase opaque cells of *V. vulnificus* biotype 2 in an ASW microcosm maintained at 25°C. ■, plate counts on MSWYE agar; ●, direct viable counts by the Kogure et al. (16) method; ▲, acridine orange direct counts.

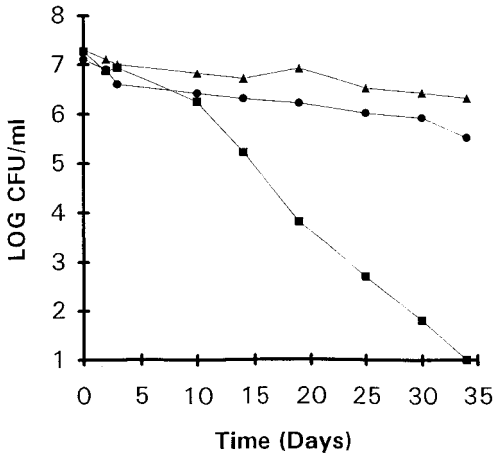

FIG. 2. Effect of low temperature (5°C) on the development of the nonculturable state of stationary-phase opaque cells of *V. vulnificus* biotype 2 in an ASW microcosm. ■, plate counts on MSWYE agar; ●, direct viable counts by the Kogure et al. (16) method; ▲, acridine orange direct counts.

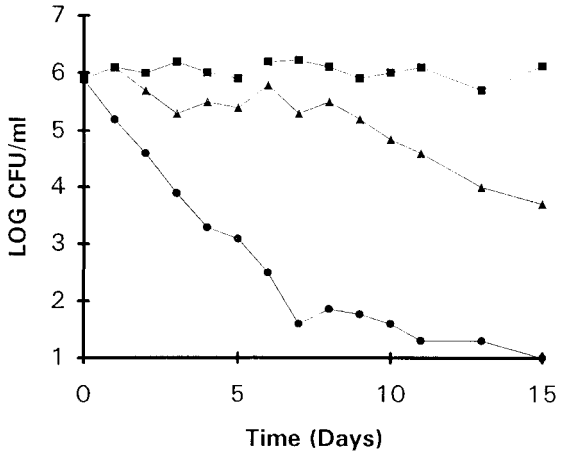

FIG. 3. Effect of prestarvation on the nonculturable response of logarithmic-phase opaque cells of *V. vulnificus* biotype 2 in ASW. Plate counts on MSWYE agar of cells incubated at 25°C (■), at 5°C (●), and at 5°C after prestarvation at 25°C for 48 h (▲) are indicated.

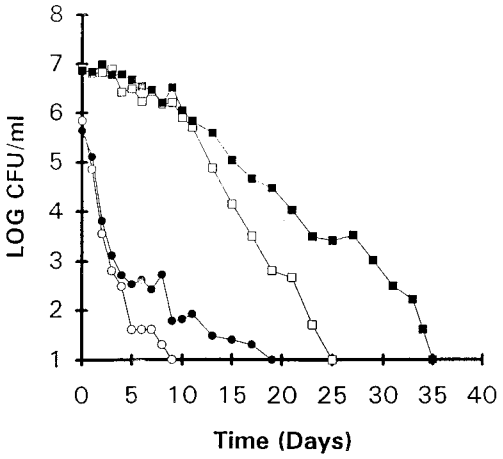

FIG. 5. Effects of growth phase (squares, stationary; circles, logarithmic) and the culture medium (open symbols, HI agar; closed symbols, MSWYE agar) on the culturability of translucent cells of *V. vulnificus* biotype 2 in an ASW microcosm at 5°C.

response similar to those observed for the eel isolate (data not shown).

We also investigated possible variations in the rate of nonculturability of *V. vulnificus* biotype 2 cells due to factors such as prestarvation, the phase of growth, and capsule expression. Logarithmic-growth-phase cells prestarved for 48 h showed a delayed response to low temperature by mantaining around 10^4 cells per ml for 15 days, whereas the number of nonprestarved cells fell to less than 10^1 cells per ml in the same period (Fig. 3). Thus, prestarved cells were more low-temperature tolerant than their nonprestarved counterparts. As shown in Fig. 4, the rate at which cells became nonculturable was dependent on their physiological age. That is, a significant difference in time to nonculturability between cells taken from the logarithmic growth phase and cells taken from the stationary phase was observed. Figure 4 shows that cells taken from stationary phase (optical density at 610 nm, 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 cells in the logarithmic growth phase (optical density at 610 nm, 0.15). To further define the sensitivity of *V. vulnificus* biotype 2 to low temperatures, the role of the capsule in survival of this microorganism was also investigated by using opaque and translucent colony variants for each strain. Survival curves demonstrated similar responses in the two morphotypes of biotype 2 cells in logarithmic and stationary phases (Fig. 4 and 5), suggesting that capsular material is not necessary for the survival of biotype 2 in water, at least under the conditions employed in our study.

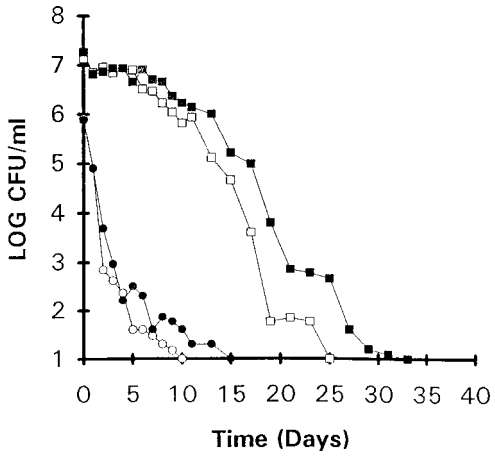

FIG. 4. Effects of growth phase (squares, stationary; circles, logarithmic) and the culture medium (open symbols, HI agar; closed symbols, MSWYE agar) on the culturability of opaque cells of *V. vulnificus* biotype 2 in an ASW microcosm at 5°C.

In all cases, culturability remained the highest on MSWYE agar, which is closer in salt and nutrient composition to ASW than is HI agar (Fig. 4 and 5). This medium-dependent difference in the growth capability on solid media was not noticeable initially but increased throughout incubation, with a greater reduction in the number of colonies recovered on HI agar than MSWYE agar (Fig. 4 and 5). A similar response was observed with the biotype 1 clinical isolate, indicating that the two biotypes of *V. vulnificus* also exhibit similar behaviors regarding the survival strategy in microcosms maintained at low temperatures (Fig. 6).

Resuscitation of nonculturable cells. As shown in Fig. 7, the inability to grow on solid media as a result of the stress conditions imposed by low temperatures was reversible in the majority of cases. In fact, simply shifting the microcosms to room temperature (arrow in Fig. 7) was enough to induce the recovery of dormant cells after 24 to 72 h of incubation. The reactivation process, induced only by a temperature shift, allowed recovery of culturable cells in numbers similar to those obtained in starved microcosms kept at room temperature the entire experiment. Moreover, after resuscitation, cells recovered their original morphology, with sizes similar to those of starved cells at 25°C. The resuscitation, recovery, or regrowth of biotype 2 cells failed in some cases (i) when small subsamples from the original microcosm were used and (ii) depending on the length of the time after entering in the dormant state (data not shown). There were no major differences in the rates of "resuscitation" between cells on MSWYE or HI plates, between opaque and translucent cells (Fig. 7), or between cells in logarithmic or stationary phase.

Virulence. The LD_{50}s of *V. vulnificus* biotype 2 unstressed cells were 4.9×10^5 and 2.6×10^2 CFU per mouse and eel,

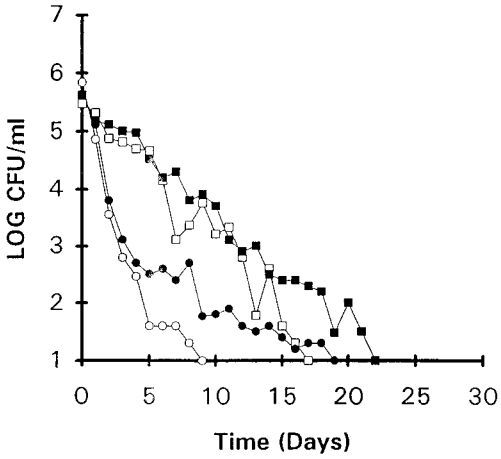

FIG. 6. Comparison of the nonculturable state of logarithmic-phase opaque cells of *V. vulnificus* biotype 1 (C7184) (squares) and biotype 2 (E22) (circles) in ASW microcosms at 5°C. Plate counts were obtained on HI agar (open symbols) and MSWYE agar (closed symbols).

respectively. Since cell enumeration by CFU was impossible with dormant cells, we used viable counts for challenge experiments with nonculturable cells because viable cells are those which might retain their virulence capability. The viable bacterial number, established by the Kogure method, of opaque cells after 1 week in the dormant state was 9.8×10^4 cells per ml, which means that the injection of 0.2 ml per mouse or 0.1 ml per eel would equal 2×10^4 and 9.8×10^3 viable cells per mouse and eel, respectively. No deaths were registered among groups of four mice or six fish injected intraperitoneally with these doses of biotype 2 cells, even after iron pretreatment with the siderophore Desferal. At this point, starved cells at room temperature maintained culturable-cell numbers around 5×10^5/ml. Such starved cells retained their infective capability for fish, with a lethal dose similar to that obtained with nonstarved cells (5.2×10^2 CFU per eel). Because the inoculation of 0.2 ml of these cells (around 10^5 CFU) per mouse was less than the lethal dose of this strain, mice were also inoculated with Desferal, resulting in 100% death. No mortalities were recorded in the control groups of mice or fish injected with ASW or PBS supplemented with Desferal.

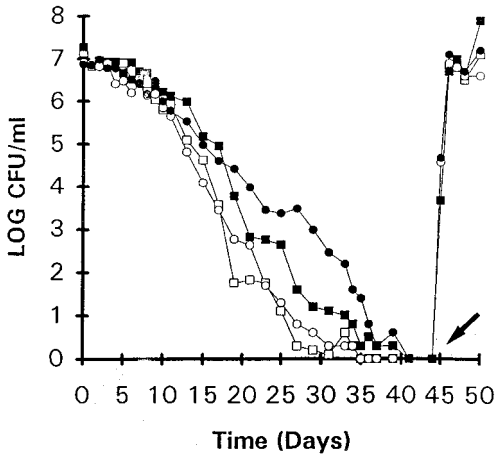

FIG. 7. Comparison of the nonculturable state and subsequent recovery following a temperature upshift of stationary-phase (squares, opaque; circles, translucent) cells of *V. vulnificus* biotype 2 in ASW microcosms at 5°C. Plate counts were obtained on HI agar (open symbols) and MSWYE agar (closed symbols). Arrow, shift of the microcosm to room temperature.

Since, like biotype 1 cells, the capsule is essential for mouse virulence of biotype 2 cells (5, 11, 30), to investigate the role of the capsule in the virulence of biotype 2 cells in the nonculturable state, translucent variants were inoculated with and without Desferal in eels (8.6×10^3 viable cells per fish). No differences were observed between opaque and translucent cells of *V. vulnificus* biotype 2, both being avirulent in the dormant state, at least under the assayed conditions.

DISCUSSION

To the best of our knowledge, no reports exist on the isolation of the eel pathogen *V. vulnificus* biotype 2 from water samples, so that the behavior of this organism outside its natural host is practically unknown. From our previous studies we know that biotype 2 is able to use water as a route of infection (4), but the hypothesis of the aquatic environment being a possible reservoir for this pathogen remains to be clarified. In an effort to address this issue, we have studied the survival of this bacterium in experimental microcosms, as well as the maintenance of its infectivity in these conditions.

In the starvation experiments conducted, we have found that cells of *V. vulnificus* biotype 2 are able to survive in the culturable state in ASW at 25°C at least for 50 days, as did the biotype 1 strain used as a control. These results indicate the capability of biotype 2 to survive in brackish or marine environments of warm temperatures, conditions which coincide with the field data recorded during the outbreaks of diseased eels in Spain (7, 8, 11). Although some other factors may be even more important than starvation for the persistence and spread of this bacterium in natural waters, this finding could explain the reappearance of the pathogen in the same farm in widely separated periods (2, 7, 11).

Some studies of *V. vulnificus* biotype 1 suggest that temperature and salinity are controlling factors in its distribution in the environment (22, 32). Moreover, low temperatures have been shown to be detrimental to survival and induce formation of viable but nonculturable cells (23, 27, 37). Therefore, we investigated the effect of low temperature on induction of dormant cells in this pathogen. *V. vulnificus* biotype 2 enters into the viable but nonculturable state when exposed to low temperature, giving a nonculturable response quite similar to the one observed for biotype 1 (27, 37). In this state, this bacterium survives for at least 50 days at low temperature, although with reduced rates of activity. These results give support to our view that biotype 2 is an aquatic bacterium that can survive in a dormant state in the environment during winter months.

In general, during starvation conditions bacterial size decreased, but the reduction was more pronounced at 5°C. This response was similar to the one observed for the biotype 1 strain used as control and to those reported previously for this biotype (17, 20). Cells of biotype 2 became nonculturable in a variable period of time depending on the conditions assayed. The main factors responsible for this variation were (i) the physiological age of the cells, because bacteria in logarithmic phase were more sensitive to cold, and (ii) prestarvation of cells, because it offers temporal protection against low temperatures. These results were observed with both the biotype 1 and the biotype 2 strains examined here and agree with those previously reported for biotype 1 (27). It has been suggested that the synthesis of carbon starvation-induced proteins (Sti) during starvation might allow for transient protection of cells

against formation of the dormant state (27, 36). Some of these proteins have been implicated in antigenic changes (1), but the surface antigens of starved cells of the biotype 2 strain E22 must be conserved, as immunological detection was achieved (unpublished data).

V. vulnificus biotype 2 exhibits distinct encapsulated (opaque) and unencapsulated (translucent) cells, due to the presence or absence of surface polysaccharidic material (10). Spanish biotype 2 isolates from diseased eels were recovered originally as opaque cells, whereas the translucent variant from each strain was obtained only after subculturing (10). Although both morphotypes are virulent for eels by intraperitoneal injection (10), only opaque cells are infective by immersion, indicating that the capsule must be essential for water-borne transmission of this pathogen (4). Thus, we have considered the possible effects of the presence or absence of the capsule in the survival strategy of biotype 2. To this end, we examined the two morphotypes and their response to starvation and low temperature in either the log growth phase or the stationary growth phase. No significant differences were found between opaque and translucent colony variants of biotype 2 cells from either the logarithmic or the stationary phase held at 5 and 25°C. Moreover, observation of colonies in plate counts taken from microcosms revealed that reversion rarely occurred under the starvation stress, indicating that the capsule was retained. The overall data suggest that the capsule is not necessary for the survival of biotype 2 in aquatic environments as a free-living organism.

Throughout this study, culturability remained the highest on MSWYE agar, which is closer in salt and nutrient composition to ASW than HI agar, both qualitatively and quantitatively. This finding indicates that *V. vulnificus* biotype 2 shows an adaptation to low-nutrient-concentration conditions, which has been shown to allow long-term survival. This fact should be kept in mind when trying to recover this bacterium from environmental waters.

Biotype 2 cells recovered their culturability on solid media after an increase of incubation temperature from 5 to 25°C, as did the biotype 1 strain used as control. This reversion, due to a shift to a favorable temperature, has been previously described for biotype 1 by Nilsson et al. (20). Temperature activation of dormant *V. vulnificus* cells has often been cited as evidence for a viable but nonculturable state. However, the reversion of dormant biotype 2 cells due to the upshift in temperature occurred only when a minimum volume of the sample was tested and a limited period existed after entering in the nonculturable state, agreeing with previous reports on this species (36). Because of the limits of detection of the plating methodology employed, it is possible that a few culturable cells remained in our microcosms and led to the apparent resuscitation observed when larger sample volumes were tested. Although further studies are necessary, our preliminary results are in accordance with the recent proposals of Weichart et al. (36) and Firth et al. (13) suggesting that temperature-dependent resuscitation of starved *V. vulnificus* biotype 1 cells is due to regrowth rather than activation of preexisting dormant cells. It is also possible that our inability to culture dormant cells under laboratory conditions may reflect the development of a form with altered nutritional or physicochemical requirements which might need special conditions for growth, as suggested by Weichart et al. (36). Indeed, the recent report by Oliver et al. (26) on entry into, and resuscitation from, the viable but nonculturable state by *V. vulnificus* when placed in natural estuarine waters, suggests that this may, in fact, be the case. Thus, we cannot preclude the possibility that reactivation of such cells can occur in aquatic environments, allowing the persistence and dissemination of this bacterium.

The first virulence experiments with dormant biotype 1 cells showed a loss of pathogenicity for mice, even in iron-pretreated animals (17), but recent studies have shown that this human pathogen retains virulence in the nonculturable state (24). In the present study, we have observed that starved cells of biotype 2 keep a virulence degree similar to that of nonstarved cells. However, dormant cells were avirulent for either eels or mice, independently of the cell morphotype tested, under the experimental conditions used here. Thus, the loss of infectivity of biotype 2 cells after entry into the nonculturable state seems to be due to factors other than loss of the capsule. In fact, the loss of virulence was observed only in cells suffering low-temperature stress. Eels are warm-water fish (14), which are cultured at around 25°C, so biotype 2 cells can likely remain in a viable and culturable state in the water of culture tanks, maintaining water-borne infectivity.

Since biotype 2 cells have shown a survival response similar to the one in biotype 1 cells, there is still the question of why it has never been recovered from natural waters. From our previous studies we know that the two biotypes of this species show a high phenotypic similarity, with indole production being the only biochemical trait that seems to differentiate them (7–9, 11). Some human septicemic cases caused by indole-negative strains have been reported in countries around the North Sea (19, 33–35), with the mode of transmission in one case being contact with brackish water (33). As a consequence of this, the lack of reports of biotype 2 from water samples may be due to its misidentification as biotype 1 (7, 9, 11), as we have recently suggested (3).

In conclusion, our results clearly indicate that *V. vulnificus* biotype 2 survives for almost 2 months in the culturable state in ASW as a free-living form. This organism is probably an autochthonous species in the marine environment, although its survival and infectivity appear to be dependent on a warm temperature. Thus, in contrast to previous reports (6), *V. vulnificus* biotype 2 seems to be not an obligate pathogen for eels but a primary pathogen able to survive in the environment away from its natural host. These results reinforce our recent proposal (4) that suggests that the aquatic environment is a reservoir and vehicle of transmission of this eel pathogen. When conditions are favorable, it may multiply in brackish or marine waters until a number sufficient enough to cause infection is reached. Thus, *V. vulnificus* biotype 2 constitutes a health hazard for both eels and humans, and preventive measures must be developed for fish farm facilities.

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REFERENCES

1. **Albertson, N. H., E. W. Jones, and S. Kjelleberg.** 1987. The detection of starvation-specific antigens in two marine bacteria. J. Gen. Microbiol. **133:** 2225–2231.
2. **Alcaide, E. (Universidad de Valencia).** 1994. Personal communication.
3. **Amaro, C., and E. G. Biosca.** The eel pathogenic bacterium *Vibrio vulnificus* biotype 2 is also an opportunistic pathogen for humans. Submitted for publication.
4. **Amaro, C., E. G. Biosca, B. Fouz, E. Alcaide, and C. Esteve.** 1995. Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. Appl. Environ. Microbiol. **61:**1133–1137.

5. **Amaro, C., E. G. Biosca, B. Fouz, A. E. Toranzo, and E. Garay.** 1994. Role of iron, capsule and toxins in the pathogenicity of *Vibrio vulnificus* biotype 2 for mice. Infect. Immun. **62:**759–763.
6. **Austin, B., and D. A. Austin.** 1987. Vibrios, p. 263–287. *In* L. M. Laird (ed.), Bacterial fish pathogens: disease in farmed and wild fish. Ellis Horwood Limited, Chichester, United Kingdom.
7. **Biosca, E. G.** 1994. Ph.D. thesis. Universidad de Valencia, Valencia, Spain.
8. **Biosca, E. G., C. Amaro, C. Esteve, E. Alcaide, and E. Garay.** 1991. First record of *Vibrio vulnificus* biotype 2 from diseased European eel, *Anguilla anguilla* L. J. Fish Dis. **14:**103–109.
9. **Biosca, E. G., C. Esteve, E. Garay, and C. Amaro.** 1993. Evaluation of the API 20E system for the routine diagnosis of the vibriosis produced by *Vibrio vulnificus* biotype 2. J. Fish Dis. **16:**79–82.
10. **Biosca, E. G., H. Llorens, E. Garay, and C. Amaro.** 1993. Presence of a capsule in *Vibrio vulnificus* biotype 2 and its relationship to virulence for eels. Infect. Immun. **61:**1611–1618.
11. **Biosca, E. G., J. D. Oliver, and C. Amaro.** Phenotypic characterization of *Vibrio vulnificus* biotype 2; a lipopolysaccharide-based homogeneous O-serogroup within *Vibrio vulnificus* species. Submitted for publication.
12. **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.
13. **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.
14. **Gault, J.** 1986. L'élevage de l'anguille, p. 742–771. *In* G. Barnabé (ed.). Aquaculture, vol. 2. Technique et Documentation (Lavoisier), Paris.
15. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44:**1246–1247.
16. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
17. **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.
18. **Mardén, P., M. Hermansson, and S. Kjellerberg.** 1986. Incorporation of tritiated thymidine by marine bacterial isolates when undergoing a starvation survival response. Arch. Microbiol. **149:**427–432.
19. **Mertens, A., J. Nagler, W. Hansen, and E. Gepts-Friedenreich.** 1979. Halophilic lactose-positive *Vibrio* in a case of fatal septicemia. J. Clin. Microbiol. **9:**233–235.
20. **Nilsson, L., J. D. Oliver, and S. Kjellerberg.** 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J. Bacteriol. **173:**5054–5059.
21. **Oliver, J. D.** 1987. Heterotrophic bacterial populations of the Black Sea. Biol. Oceanogr. **4:**83–97.
22. **Oliver, J. D.** 1989. *Vibrio vulnificus*, p. 570–600. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, Inc., New York.
23. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239–272. *In* S. Kjellerberg (ed.), Starvation in bacteria. Plenum Press, New York.
24. **Oliver, J. D., and R. Bockian.** 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl. Environ. Microbiol. **61:**2620–2623.
25. **Oliver, J. D., and R. R. Colwell.** 1973. Extractable lipids of gram-negative marine bacteria: phospholipid composition. J. Bacteriol. **114:**897–908.
26. **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*. Appl. Environ. Microbiol. **61:**2624–2630.
27. **Oliver, J. D., L. Nilsson, and S. Kjellerberg.** 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Appl. Environ. Microbiol. **57:**2640–2644.
28. **Reed, M. J., and M. Münch.** 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
29. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
30. **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.
31. **Song, Y.-L., W. Cheng, C.-H. Shen, Y.-C. Ou, and H.-B. Song.** 1990. Occurrence of *Vibrio vulnificus* in cultured shrimp and eel in Taiwan, p. 172–179. *In* Proceedings of the ROC-Japan Symposium on Fish Diseases.
32. **Tamplin, M. L., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. **44:**1466–1470.
33. **Veenstra, J., P. J. G. M. Rietra, J. M. Coster, E. Slaats, and S. Dirsks-Go.** 1994. Seasonal variations in the occurrence of *Vibrio vulnificus* along the Dutch coast. Epidemiol. Infect. **112:**285–290.
34. **Veenstra, J., P. J. G. M. Rietra, J. Goudswaard, J. A. Kaan Slaats, P. H. J. van Keulen, and C. P. Stoutenbeek.** 1993. Extra intestinale infecties door *Vibrio* spp. in Netherland. Ned. Tijdschr. Geneeskd. **138:**654–657.
35. **Veenstra, J., P. J. G. M. Rietra, C. P. Stoutenbeek, J. M. Coster, H. H. W. De Hier, and S. Dirsks-Go.** 1992. Infection by an indole-negative variant of *Vibrio vulnificus* transmitted by eel. J. Infect. Dis. **16:**209–210.
36. **Weichart, D., J. D. Oliver, and S. Kjellerberg.** 1992. Low temperature induced nonculturability and killing of *Vibrio vulnificus*. FEMS Microbiol. Lett. **100:**205–210.
37. **Wolf, P. W., and J. D. Oliver.** 1992. Temperature effects on the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol. Ecol. **101:**33–39.
38. **Wright, A. C., L. M. Simpson, and J. D. Oliver.** 1981. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect. Immun. **34:**503–507.
39. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Atwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of the non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8:**313–323.