Temperature-induced recovery of *Vibrio cholerae* from the viable but nonculturable state: growth or resuscitation?

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***Vibrio cholerae* cells were incubated at 4 °C in nutrient-limited artificial seawater (ASW) microcosms. Plate counts declined from 8×10^5 to less than 2 c.f.u. ml⁻¹ in about 23 d. When samples of microcosms were shifted to 30 °C, plate counts increased to 2.2×10^5 c.f.u. ml⁻¹ in 72 h. An experiment was performed to determine whether culturable cells obtained after temperature upshifts were the result of 'resuscitation', or outgrowth, of nonculturable cells or of cell division and growth of the few culturable cells that remained in samples. Prior to temperature upshift, samples from the microcosms were diluted 10- and 100-fold in filter-sterilized (0.1 µm) ASW from the microcosms. Undiluted, 1/10, and 1/100 diluted samples recovered culturability to about 2.2×10^5 c.f.u. ml⁻¹ within 72 h of temperature upshift. If resuscitation of nonculturable cells had occurred, the resultant number of culturable cells in diluted samples would have been 1/10 and 1/100 that of undiluted samples, respectively. In microcosms where plate counts had declined to less than 1 c.f.u. ml⁻¹, 1/100 diluted samples did not regain culturability, i.e. no culturable cells remained from which growth could occur. Our conclusions are that in the experiments reported here, recovery of culturable cells on temperature upshifts resulted from growth and that there were no growth-inhibiting factors in the spent growth medium, supported by the finding that about 10^2 recovered *V. cholerae* cells ml⁻¹ inoculated into filter-sterilized microcosm ASW grew to about 6.2×10^5 c.f.u. ml⁻¹ in 24 h, confirming that *V. cholerae* is capable of significant growth in ASW.**

Keywords: *Vibrio cholerae*, viable but nonculturable cells, regrowth, resuscitation

INTRODUCTION

Discoveries of the past decade have revealed the existence of a dormant state into which *Vibrio cholerae* enters, in response to environmental parameters such as temperature and salinity (Xu *et al.*, 1982), and nutrient deprivation (Baker *et al.*, 1983). Colwell and co-workers have suggested that *V. cholerae* can enter a state where the cells are viable but nonculturable (Xu *et al.*, 1982; Brayton & Colwell, 1987; Colwell *et al.*, 1990). In this state, cells are reduced in size, become ovoid and, in contrast to starved cells, cannot be grown using conventional bacteriological techniques. The existence of a viable but nonculturable

state, in response to adverse environmental conditions, has been observed for Gram-negative bacteria, for example, *Escherichia coli* (Xu *et al.*, 1982; Byrd & Colwell, 1990), *Vibrio vulnificus* (Linder & Oliver, 1989; Nilsson *et al.*, 1991), *Salmonella enteritidis* (Roszak & Colwell, 1987), *Aeromonas salmonicida* (Allen-Austin *et al.*, 1984), *Shigella sonnei* and *Shigella flexneri* (Colwell *et al.*, 1985), *Shigella dysenteriae* (Islam *et al.*, 1993; Rahman *et al.*, 1994), *Helicobacter* (*Campylobacter*) *jejuni* (Rollins & Colwell, 1986), and *Helicobacter pylori* (Shahamat *et al.*, 1993).

Methods described by Kogure *et al.* (1979) and Zimmermann *et al.* (1978) are useful in demonstrating that dormant cells retain viability, even though these cells are nonculturable on conventional media. It has been concluded that the phenomenon is a bacterial survival mechanism, with entry into the viable but nonculturable

Abbreviations: AODC, acridine orange direct counts; ASW, artificial seawater; DVC, direct viable counts.

state being influenced by environmental parameters such as temperature, salinity and nutrient concentration, with species and strain variations also being reported (Roszak & Colwell, 1987). Moreover, it has been shown that viable but nonculturable cells remain potentially pathogenic. The existence of a viable but nonculturable state for *V. cholerae* may be an important and fundamental ecological consideration in worldwide epidemics of *V. cholerae* (Colwell *et al.*, 1990; Epstein *et al.*, 1993).

Inability to detect viable cells by conventional microbiological plating or inoculation into enrichment media has stimulated investigations on recovery of cells from the viable but nonculturable state. Addition of nutrient was used by Roszak *et al.* (1984) to recover *S. enteritidis*. Recently, Kaprelyants & Kell (1993) used rich liquid medium to recover nonculturable *Micrococcus luteus* cells from long-term storage in spent growth medium and using flow cytometric analysis concluded that resuscitation of *M. luteus* had occurred. Nilsson *et al.* (1991) reported that the estuarine pathogenic bacterium *V. vulnificus* can recover from the viable but nonculturable state, without nutrient addition, by responding to a temperature upshift from 4 °C to room temperature (23 °C). They concluded that this temperature upshift induced nonculturable cells to recover from the viable but nonculturable state to the original cell number. Nilsson *et al.* (1991) concluded that recovery of culturable cells was due to resuscitation of these cells rather than growth of a few remaining culturable cells.

Following the experimental protocol of Nilsson *et al.* (1991), we have now shown that viable but nonculturable *V. cholerae* cells underwent the same 'recovery' when the incubation temperature was shifted up to 30 °C after incubation at 4 °C. However, we also performed experiments to distinguish between true resuscitation and apparent resuscitation resulting from regrowth of a small number of *V. cholerae* cells that remained culturable. In addition, we investigated the ability of *V. cholerae* to grow in a very low nutrient medium.

METHODS

Strains and microcosm conditions. Two strains of *Vibrio cholerae* were used in these studies: a streptomycin-resistant derivative of *V. cholerae* 569B, and *V. cholerae* CVD101, a *tox A* mutant of *V. cholerae* 395 (Kaper *et al.*, 1984). *V. cholerae* was incubated at 37 °C in Luria-Bertani (LB) broth (Maniatis *et al.*, 1982) and harvested at an OD₆₀₀ of 0.8–1.0 by centrifugation at 1400 g for 5 min. Cells were washed sequentially at room temperature in 5% (w/v) LB (NaCl 5 g l⁻¹), 1% LB (NaCl 5 g l⁻¹), and twice in 10 p.p.t. artificial sea water (ASW) (Instant Ocean, Aquarium Systems) to prevent carry-over of nutrients on inoculation of microcosms. ASW contains undetectable concentrations of organic carbon (R. Studt, Aquarium Systems, personal communication). Cells were recovered after each wash by centrifugation (1400 g for 5 min). During this washing procedure, cells were held for 1 h in the 1% LB wash. In order to obtain an accurate cell count, 1 ml of a 10⁻⁴ dilution was stained with 100 µl 0.1% acridine orange (Sigma) and the cells were collected by filtration through a 0.2 µm black filter (Poretics) according to the procedure of Hobbie *et al.* (1977). Cells were inoculated at about 10⁵ cells ml⁻¹ in 100 ml 10 p.p.t.

ASW in pre-cooled 250 ml flasks. The flasks had been scrupulously cleaned by overnight soaking in a 5% (v/v) solution of RBS35 detergent (Pierce), followed by 30 rinses in deionized distilled water and overnight treatment in 5 M HCl, followed by a further 30 rinses. Flasks were filled with deionized distilled water, autoclaved and emptied prior to addition of sterile 10 p.p.t. ASW. The microcosms were incubated at 4 °C in the dark. Microcosms were prepared in duplicate for *V. cholerae* 569B, and in triplicate for *V. cholerae* CVD101.

Cell counts. Samples taken at the time of incubation (time 0) and at subsequent time intervals were monitored by plate counts, acridine orange direct counts (AODC) and direct viable counts (DVC). Plate counts were obtained by spreading appropriate dilutions of *V. cholerae* onto LB agar. Plates were incubated at 30 °C, and colonies were counted after 2 d. Total bacterial counts were performed by AODC, as described by Hobbie *et al.* (1977). Cells were counted at a magnification of 1000× using an Olympus Vanox AHBTEpi fluorescent microscope. Counting was done manually and approximately 300–700 bacteria per filter were counted. Direct viable counts were done by a modification of the procedure of Kogure *et al.* (1979). Samples were enriched to a final concentration of 0.25% (w/v) yeast extract in the presence of 15 µg nalidixic acid ml⁻¹ and incubated for 24 h at 30 °C in the dark with shaking. Elongated cells were counted by epifluorescent microscopy, as described for AODC. Both total counts and direct viable counts were recorded.

Microscopic observation. Morphological changes of *V. cholerae* cells were monitored as the cells entered the viable but nonculturable state at 4 °C, as well as 'recovery' at 30 °C by AODC.

Temperature upshift experiments. Triplicate microcosms of *V. cholerae* CVD101 in 10 p.p.t. ASW were prepared following the methods described above. Plate counts and AODC were monitored periodically. At eight time points (4, 7, 14, 17, 20, 24, 28 and 33 d), 10 ml subsamples were removed to sterile polypropylene 15 ml tubes for incubation at 30 °C in the dark without shaking. Plate counts in these subsamples were determined daily for 4 d, with the exception of the last two time points, which were monitored for 6 d.

Dilution and temperature upshift experiments. Microcosms were monitored until the plate counts decreased to 2 c.f.u. ml⁻¹. Ten millilitre samples of the microcosms were filtered through 0.2 µm membrane filters (Millipore), and the filters were placed 'face up' (side with collected cells) onto LB agar. Colonies were counted after 48 h. Samples from microcosms were diluted in 10 p.p.t. ASW to give 1/10 and 1/100 dilutions. The 10 p.p.t. ASW diluent was obtained from original microcosms in which the *V. cholerae* cells had entered the viable but nonculturable state and was rendered cell-free by filtration through a 0.1 µm filter. Experiments were done in a final volume of 5 ml in 50 ml sterile polypropylene tubes. The incubation temperature for the 1/10 and 1/100 dilutions and a 5 ml undiluted control was shifted to 30 °C. Tubes were incubated in the dark without shaking. Plate counts and total cell counts were monitored as described above. In order to show that the diluent used did not contain any cells which were able to recover culturability after the temperature upshift, 5 ml samples of the 0.2 µm-filtered microcosms were incubated at 30 °C, and plate counts were done. Also, the effect of the subsampling procedure on recovery was followed by maintaining and monitoring a 5 ml subsample in the dark at 4 °C.

Growth at very low nutrient concentrations. Using the method described above, 10³ or 10² cells ml⁻¹ were inoculated into 5 ml filter-sterilized 10 p.p.t. ASW. Quadruplicate micro-

cosms were incubated at 30 °C in the dark without shaking. Plate counts were monitored on LB medium for 4 d.

Ten-microlitre samples of a recovered microcosm containing 6.7×10^5 c.f.u. ml⁻¹ were inoculated into 10 ml sterile 10 p.p.t. ASW and into 0.1 µm filter-sterilized 10 p.p.t. ASW from a microcosm in which cells had entered the viable but non-culturable state. These were incubated at 30 °C for 3 d. Samples were removed periodically to determine culturability by plating on tryptic soy agar (Difco).

Reproducibility of results. Entry into the viable but non-culturable state was monitored in duplicate or triplicate microcosms and results are presented as a mean value $\pm$ SD. Dilution experiments were done in duplicate subsamples from duplicate microcosms and results shown are the mean of four subsamples. Homogeneity of the variance was calculated using the Fisher's Protected LSD test at the significance level of 0.05. Statistical analyses were performed with the SuperANOVA program (Abacus Concepts). Populations in different microcosms and subsamples were considered to be not significantly different if $P > 0.05$.

RESULTS

Viable but nonculturable state of *V. cholerae* in ASW

Microcosms were prepared in duplicate and incubated in ASW (salinity 10 p.p.t.) at 4 °C with an initial inoculum of about 10^6 cells ml⁻¹. The viable but nonculturable response of *V. cholerae* 569B is shown in Fig. 1. After 26 d, plate counts in duplicate microcosms had decreased to ≤ 1.7 c.f.u. ml⁻¹, while the total direct count increased slightly from 7.6×10^5 cells ml⁻¹ to 8.3×10^5 cells ml⁻¹. Although the culturable count was less than two culturable cells ml⁻¹, the viable count as determined by DVC (Kogure *et al.*, 1979) was still at least 2.8×10^4 cells ml⁻¹, indicating that the cells were in the viable but nonculturable state as described by Colwell *et al.* (1985).

Fig. 1. Entry of *V. cholerae* 569B into the viable but nonculturable state at low temperature (4 °C), and recovery after temperature upshift from 4 °C to 30 °C. Changes in cell morphology during the entry of *V. cholerae* into the viable but nonculturable state and recovery are shown at the top. The arrow indicates temperature upshift from 4 °C to 30 °C. ●, Total cell count determined by AODC; ■, plate counts on LB. Results are means from duplicate experiments $\pm$ SD.

Fig. 2. Plate counts of *V. cholerae* CVD101 incubated at 4 °C in 10 p.p.t. ASW (■) and recovery of mini-microcosm samples by temperature upshift from 4 °C to 30 °C (□). ●, AODC of samples from the microcosm. NG, no growth upon temperature upshift. Results are means of triplicate experiments $\pm$ SD.

Samples of these microcosms containing an average of 1.7 culturable cells ml⁻¹ were shifted from an incubation temperature of 4 °C up to 30 °C. After incubation for 3 d at 30 °C, *V. cholerae* 569B cells had recovered culturability because 3.2×10^5 cells ml⁻¹ were culturable (Fig. 1). Longer incubation at 30 °C did not increase the culturable count further and an insignificant decrease was observed from 3.2×10^5 to 2.9×10^5 c.f.u. ml⁻¹ after two more days.

Viable but nonculturable *V. cholerae* CVD101 cells showed the same response as *V. cholerae* 569B when incubated in 10 p.p.t. ASW and shifted from 4 °C to 30 °C. *V. cholerae* CVD101 cells were used in an experiment where recovery by temperature upshift was assessed at eight time points during entry into the viable but nonculturable state. Fig. 2 shows the response of *V. cholerae* CVD101 cells in microcosms when incubated in 10 p.p.t. ASW at 4 °C, and subsamples subsequently incubated at 30 °C. Cells became nonculturable after 20 d. Subsamples processed at 28 d and 33 d did not recover from the viable but nonculturable state after 6 d, an observation similar to that of H.-S. Xu & R. R. Colwell who found that much longer incubation is required (unpublished data).

Dilution and temperature upshift experiments

In order to investigate whether recovery by temperature upshift was due to true resuscitation or to growth of a few culturable cells of *V. cholerae* 569B which remained in the microcosms, dilution experiments were done. The initial number of culturable cells in microcosms was about 1.7 cells ml⁻¹ (Table 1). If resuscitation of all viable but nonculturable cells had occurred, plate counts in 1/10- and 1/100-diluted samples would be expected to recover to 1/10 and 1/100 of the level in the undiluted sample, respectively. However, growth from a small number of

Table 1. AODC and plate counts of *V. cholerae* 569B after temperature upshift from 4 °C to 30 °C for undiluted samples, and samples diluted 1/10 and 1/100 in filter-sterilized ASW

Means from undiluted samples were obtained from duplicate experiments and those from diluted samples were from quadruplicate experiments.

Time at 30 °C (h)	Undiluted		1/10 dilution		1/100 dilution	
	AODC (cells ml ⁻¹)	Plate count (c.f.u. ml ⁻¹)	AODC* (cells ml ⁻¹)	Plate count† (c.f.u. ml ⁻¹)	AODC* (cells ml ⁻¹)	Plate count† (c.f.u. ml ⁻¹)
0	8.2×10^5	1.7×10^0	8.2×10^4	1.7×10^{-1}	8.2×10^3	1.7×10^{-2}
20		0.9×10^2		4.9×10^2		6.5×10^1
49		3.2×10^5		2.4×10^5		1.4×10^5
67		2.6×10^5		2.0×10^5		2.4×10^5
91	1.9×10^6	2.9×10^5	1.8×10^6	2.1×10^5	1.7×10^6	4.3×10^5

* Values at time 0 were deduced from the AODC of the undiluted sample.

† Values at time 0 were deduced from the plate count of the undiluted sample.

Fig. 3. Plate counts of recovered *V. cholerae* CVD101 cells incubated at 30 °C in 10 p.p.t. ASW (□) and filter-sterilized microcosm 10 p.p.t. ASW (■). Results are means of duplicate experiments $\pm$ sd. **Fig. 4.** Plate counts of *V. cholerae* 569B cells incubated at 30 °C in filter-sterilized 10 p.p.t. ASW. Cells were inoculated at concentrations of about 10^2 (□) or 10^3 (■) cells ml⁻¹. Results are means of duplicate experiments $\pm$ sd.

cells that had remained culturable would give an increase in plate counts in all three samples to approximately the same number of cells, since nutrients available in each sample would be similar. Results from this experiment are shown in Table 1. After 67 h, plate counts in undiluted 1/10- and 1/100-diluted samples were all at about 2.2×10^5 c.f.u. ml⁻¹. The increase in the total cell count was similar for all three samples after incubation for 91 h at 30 °C (Table 1). Dilutions were performed in quadruplicate; and one of the 1/100-diluted samples did not recover culturability and is not included in Table 1. A similar experiment was performed with microcosms which contained about 0.1 culturable cells ml⁻¹. In this case 1/100 and 1/1000 dilutions were tested and after incubation for up to 6 d, no cells recovered culturability in these diluted samples, although the undiluted control recovered to a platable count of 2.1×10^5 c.f.u. ml⁻¹ after incubation for 3 d, and the total cell count increased from 8.9×10^5 cells ml⁻¹ to 1.5×10^6 cells ml⁻¹ in 6 d.

Growth at very low nutrient concentrations

To test growth of *V. cholerae* cells under the very low nutrient conditions present in microcosms, recovered *V. cholerae* CVD101 cells were inoculated into filter-sterilized 10 p.p.t. ASW from a microcosm in which cells had entered the viable but nonculturable state, and in fresh 10 p.p.t. ASW. Fig. 3 shows growth to 2.5×10^3 c.f.u. ml⁻¹ of recovered *V. cholerae* cells in 10 ml fresh 10 p.p.t. ASW and filtered-sterilized microcosm ASW: cell growth occurred in both media. After 24 h the plate count had reached 6.2×10^5 c.f.u. ml⁻¹, and no further increase was obtained after incubation for 3 d at 30 °C.

Growth of *V. cholerae* 569B was assessed in 10 p.p.t. ASW at 30 °C. Growth curves obtained from microcosms inoculated with 10^3 or 10^2 cells ml⁻¹ are shown in Fig. 4.

As assessed by plate counts, $1-2 \times 10^5$ c.f.u. ml⁻¹ were detectable in both microcosms after 4 d.

DISCUSSION

V. cholerae 569B cells entered the viable but nonculturable state after about 24 d when incubated at 4 °C in 10 p.p.t. ASW. In a previous study (unpublished results), *V. cholerae* was found to enter this state more rapidly at 4 °C than at 25 °C. These results and the seasonality observed in the detection of *V. cholerae* in the environment (Chowdhury *et al.*, 1992) indicate an important role of temperature and salinity for entry into the viable but nonculturable state by *V. cholerae*. Nilsson *et al.* (1991) recovered *V. vulnificus*, an estuarine bacterium, from the viable but nonculturable state, using only a temperature upshift from 4 °C to room temperature (23 °C). They described this phenomenon as 'resuscitation' from the viable but nonculturable state. This was the first report of recovery of viable but nonculturable cells without addition of nutrient, and suggested that temperature may be the single most important factor in resuscitation from the viable but nonculturable state in some species of bacteria, at least for *V. vulnificus*. Subsequently, it was shown that on incubation at low temperatures *V. vulnificus* apparently produces a factor that causes a decline in culturability, but a few cells retain the ability to grow, and the 'resuscitation' reported previously was probably regrowth from the few culturable cells, rather than resuscitation *per se* (Weichart *et al.*, 1992).

We investigated the response of *V. cholerae* to a temperature upshift from 4 °C to 30 °C. Nonculturable *V. cholerae* cells regained culturability from 1.7 c.f.u. ml⁻¹ to 3.2×10^5 c.f.u. ml⁻¹, after incubation for 3 d at 30 °C (Fig. 1). These results were similar to those obtained by Nilsson *et al.* (1991), who showed that even if *V. vulnificus* did not respond to nutrient downshift like *V. cholerae* at 25 °C (Wolf & Oliver, 1992), both organisms recovered from the viable but nonculturable state after only a temperature upshift from 4 °C to room temperature. Changes in morphology of *V. cholerae* cells observed during the entry into the viable but nonculturable state in this study are similar to those published more than ten years ago (Xu *et al.*, 1982; Baker *et al.*, 1983) and to those reported for *V. vulnificus* (Nilsson *et al.*, 1991). After incubation in very low nutrient medium (10 p.p.t. ASW), *V. cholerae* underwent morphological change and typical comma-shaped cells transformed into small cocci as shown in Fig. 1. However, upon resuscitation, none of the *V. cholerae* cells reverted back to the original rod shape, but remained coccoid, although a slight increase in size was observed (Fig. 1).

Methods that have been proposed to enumerate viable cells include the reduction of *p*-iodonitrotetrazolium violet (INT) to INT-formazan by the electron transport system of metabolizing cells (Zimmermann *et al.*, 1978) and the DVC method (Kogure *et al.*, 1979). A problem with the INT assay is that it is difficult to detect formazan crystals in the very small, viable but nonculturable cells, such as those observed in this study. In the DVC method,

yeast extract and nalidixic acid are added to samples and after incubation elongated cells can be readily observed and are counted as viable cells. This method gives an estimate, through direct examination, of the number of viable cells in a population and is convenient to use when processing many samples. However, in some cases, as in this study, the bacteria appeared only slightly swollen but not clearly elongated. It is likely that under certain conditions the DVC method underestimates the number of viable cells present. Variability in response, based on concentration of yeast extract and nalidixic acid, has been reported (Roszak *et al.*, 1984), and a study of this very point was made by Peele & Colwell (1981). A method using the fluorescent redox probe 5-cyano-2,3-ditolyl-tetrazolium chloride (Rodriguez *et al.*, 1992) is presently under investigation for detection of viable *V. cholerae* cells in laboratory microcosms and appears to give good results.

Because it was unclear, in our view, whether the *V. cholerae* cells did indeed revert to the culturable state by temperature upshift alone, we investigated whether *V. cholerae* cells were responsive to temperature upshift throughout entry into the viable but nonculturable state. *V. cholerae* cells recovered from the viable but nonculturable state at all time points tested (Fig. 2). However, after 8 d in the nonculturable state (28 d after the commencement of the experiment), no cells were recovered after temperature upshift. One explanation for this observation is that no culturable cells were present in the subsample mini-microcosm taken at these particular time points. This suggested the hypothesis, that in such cases, a few culturable cells are necessary in order to observe recovery from the viable but nonculturable state, and indicated that cell division from a few remaining culturable cells may be a requirement for recovery of culturable cells after temperature upshift.

Thus, experiments were designed explicitly to determine whether recovery from temperature upshift was due to resuscitation or to division of the few culturable cells that remained. Samples of nonculturable microcosms were diluted in filter-sterilized microcosm 10 p.p.t. ASW. The same number of culturable cells was obtained after temperature upshift in 10- and 100-fold dilutions and in undiluted control samples after incubation for 2 d at 30 °C, indicating that growth had occurred during the process of recovery and that there was no inhibiting factor in the spent growth medium. This experiment demonstrated that growth occurred during the temperature upshift, but does not exclude the possibility that resuscitation of viable but nonculturable cells occurred as the first step in the recovery process. Cells could recover by reverting to a culturable state by resuscitation and then grow to reach the maximum carrying capacity of the medium, in this case about 2.4×10^5 c.f.u. ml⁻¹. However, when samples from a microcosm where only 0.2 cells ml⁻¹ were culturable were diluted 100-fold, no recovery of culturable cells was observed after 6 d. Furthermore, one of the quadruplicate samples in the initial dilution experiment did not recover, possibly because no culturable cells remained in this particular sample, i.e.

extinction dilution occurred. Thus, it is concluded from this study with *V. cholerae* that the presence of a small number of culturable cells is required for recovery to occur by temperature upshift alone.

In some cases, recovery occurred from dilutions in which less than one culturable cell was calculated to be present. For example, in the experiment where about 1.7 culturable cells were present in undiluted samples, only about 0.085 culturable cells should have been present in 100-fold dilutions (of 5 ml volume), yet recovery was obtained in three out of four cases (Table 1). The reason for this is not clear, but one possible explanation is that cells just on the transition of entry into the viable but nonculturable state are not platable on direct transfer from liquid medium at 4 °C to solid medium at room temperature, but can be plated when held at room temperature in liquid medium prior to plating. Another possibility is that a population of cells is present that is nonculturable on solid medium, but which can grow and divide in liquid medium, after temperature upshift. This would also explain the discrepancy between the increase in AODC and the increase in plate counts in the 10- and 100-fold dilutions in Table 1. For example, plate counts in the 100-fold dilution increased from 1.7×10^{-2} to 4.3×10^5 (an increase of about 4.3×10^5) whereas AODCs increased from 8.2×10^3 to 1.7×10^6 (an increase of about 1.7×10^6). If any population of cells is present that is nonculturable on solid medium, plate counts may underestimate the total growth that has occurred and these may have been accurately measured by AODC. This does not alter our conclusion that recovery of culturable cells from temperature upshift alone appears to be dependent on cell division and growth, and not solely the result of resuscitation of viable but nonculturable cells.

Additional evidence supporting this conclusion was obtained from experiments on growth of *V. cholerae* under very low nutrient conditions. *V. cholerae* cells which had recovered after temperature upshift were inoculated, at a concentration of 2.4×10^2 c.f.u. ml⁻¹, in 10 p.p.t. ASW and filter-sterilized microcosm 10 p.p.t. ASW, and grew to 6.2×10^5 c.f.u. ml⁻¹ (Fig. 3). The observation that *V. cholerae* could undergo cell division in a very low nutrient medium to exhibit a thousandfold increase in cell numbers supports our conclusion that cell division occurred during recovery to culturability, after the temperature upshift. Moreover, fresh culturable *V. cholerae* cells grew to a concentration of about 10^5 c.f.u. ml⁻¹ when this strain was inoculated at low cell densities (10^2 – 10^3 cells ml⁻¹) in 10 p.p.t. ASW at 30 °C (Fig. 4).

Since recovery of viable but nonculturable cells was not achieved when all the cells were nonculturable (i.e. after incubation for a further 8 d after no culturable cells were detectable, despite temperature upshift) we conclude that recovery of culturable cells when viable but nonculturable *V. cholerae* cells are subjected to a temperature upshift alone from 4 °C to 30 °C may be a result of the regrowth of the few culturable cells which survive and remain in the medium, and not resuscitation of *all cells* from the viable but nonculturable state. The possibility has not been eliminated that resuscitation of a small number of

cells occurred, and was followed by growth of these resuscitated cells. It is also possible that a resuscitation-inducing factor may be produced by culturable cells that triggers resuscitation in nonculturable cells. Kaprelyants *et al.* (1994) found that dormant *Micrococcus luteus* cells were resuscitated, apparently by a factor produced by viable (platable) cells, an observation meriting further study.

Concluding remarks

An understanding of the viable but nonculturable state in *V. cholerae* is of critical importance for the purpose of monitoring the aquatic environment for this pathogen. This study indicates that, although important, temperature change alone is insufficient to bring about recovery of *V. cholerae* from the viable but nonculturable state. Hence, interpretation of results of temperature-based experiments require careful examination and interpretation. Recent work with *Helicobacter pylori* in our laboratory suggests that a combination of factors, including temperature and nutrient concentration, play a significant role either in combination and/or in relation to other environmental factors in recoverability from the viable but nonculturable state (Mai *et al.*, 1990). Obviously, the physiology and molecular genetic regulation of the viable but nonculturable state in Gram-negative bacteria such as *V. cholerae* need to be elucidated.

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