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ORIGINAL ARTICLE

Recovery in culture of viable but nonculturable *Vibrio parahaemolyticus*: regrowth or resuscitation?

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The objective of this study was to explore the recovery of culturability of viable but nonculturable (VBNC) *Vibrio parahaemolyticus* after temperature upshift and to determine whether regrowth or resuscitation occurred. A clinical strain of *V. parahaemolyticus* Vp5 was rendered VBNC by exposure to artificial seawater (ASW) at 4°C. Aliquots of the ASW suspension of cells (0.1, 1 and 10 ml) were subjected to increased temperatures of 20°C and 37°C. Culturability of the cells in the aliquots was monitored for colony formation on a rich medium and changes in morphology were measured by scanning (SEM) and transmission (TEM) electron microscopy. Samples of VBNC cells were fixed and examined by SEM, revealing a heterogeneous population comprising small cells and larger, flattened cells. Forty-eight hours after temperature upshift to 20°C or 37°C, both elongation and division by binary fission of the cells were observed, employing SEM and TEM, but only in the 10-ml aliquots. The results suggest that a portion of VBNC cells is able to undergo cell division. It is concluded that a portion of VBNC cells of *V. parahaemolyticus* subjected to cold temperatures remain viable. After temperature upshift, regrowth of those cells, rather than resuscitation of all bacteria of the initial inoculum, appears to be responsible for recovery of culturability of VBNC cells of *V. parahaemolyticus*. Nutrient in filtrates of VBNC cells is hypothesized to allow growth of the temperature-responsive cells, with cell division occurring via binary fission, but also including an atypical, asymmetric cell division.

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Introduction

In the natural aquatic environment, many Gram-negative bacteria, that is, *Vibrio* spp., *Salmonella* spp., *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli*, *Shigella dysenteriae*, *Pasteurella piscicida*, *Pseudomonas fluorescens*, *Streptococcus pyogenes*, *Aeromonas salmonicidae* and *Helicobacter pylori*, and some Gram-positive bacteria like *Enterococcus faecalis* are no longer culturable when exposed to conditions adverse to growth (Gauthier, 2000). The loss of culturability, accompanied by maintenance of metabolic activity, is considered to reflect entry of the cells into a viable but nonculturable (VBNC) state. This VBNC state, proposed by Roszak *et al.* (1984), was first demonstrated by Xu *et al.* (1982). This physiological state describes the inability of a bacterial cell to grow on media normally supporting growth of the bacterium under laboratory conditions. To detect viability of these cells, several methods were developed. For example, the direct viable count method (Kogure *et al.*, 1979) determines whether these bacteria retain the ability to enlarge in the presence of nutrient and nalidixic acid. Other methods determine whether respiration occurs, by measuring reduction of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl

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tetrazolium chloride) (Zimmermann *et al*., 1978) or CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (Rodriguez *et al*., 1992), or by expression of proteins, employing microradiography (Rahman *et al*., 1994). Yet another method is detection of transcription by reverse transcriptase-polymerase chain reaction (PCR). The half-life of mRNA being very short, it is considered to be a good marker for viability and has been used with success, both *in vitro* and *in situ* studies (Sheridan *et al*., 1998; Lleo *et al*., 2000; Fischer-Le Saux *et al*., 2002; Yaron and Matthews, 2002; Coutard *et al*., 2005; Smith and Oliver, 2006).

Vibrio parahaemolyticus is an estuarine bacterium that is also a pathogen associated with seafood, causing gastrointestinal disease with characteristic symptoms such as vomiting, diarrhea, abdominal cramps and fever. The frequency of isolation of *V. parahaemolyticus* from environmental samples is much lower during winter than summer (Chowdhury *et al*., 1990). VBNC *V. parahaemolyticus* can be induced by exposing cells to low temperatures (Jiang and Chai, 1996; Mizunoe *et al*., 2000). In terms of public health, an important consideration is the risk posed by these bacteria when they cannot be detected by classical methods, especially in samples collected during winter, and whether they are able to recover culturability under appropriate conditions. The keystone of the VBNC hypothesis, therefore, is demonstration of recovery of culturability of VBNC cells. However, whether recovery of culturability of such cells occurs has long been debated. Although several studies have suggested that the culturable population may be the result of growth of a very few cells remaining culturable (Ravel *et al*., 1995; Jiang and Chai, 1996), others have concluded that nonculturable bacteria should be able to revert to the culturable state, rather than undergo reductive cell division, characteristic of cells that have undergone starvation (Oliver *et al*., 1991). Hypotheses to explain recovery of culturability of a VBNC population include the following: either all bacteria in the original inoculum are in the VBNC state and are able to recover culturability by resuscitation (Nilsson *et al*., 1991; Whitesides and Oliver, 1997), or only a few of the bacteria are VBNC, maintaining the ability to regrow, thereby mimicking resuscitation of the entire population (Weichart *et al*., 1992). Resuscitation of VBNC cells was observed by Nilsson *et al.* (1991) in the case of *Vibrio vulnificus*, and this finding was interpreted that all of the bacteria became VBNC. That is, the cells of the bacterial population reacted homogeneously and all became VBNC and resuscitated without cell division.

The objective of this study was to determine if VBNC *V. parahaemolyticus* returned to culturability after temperature upshift resulting from resuscitation of the entire bacterial population or rapid growth of a sub-population of the VBNC cells.

Materials and methods

Bacteriological strains and culture media

V. parahaemolyticus Vp5, isolated from a clinical sample, was cultured on Heart Infusion Agar (HIA; Difco Laboratories, Detroit, MI, USA). A single colony was inoculated in 20 ml of HI broth and incubated overnight at 37°C, with shaking (70 r.p.m.), in the dark. Decimal dilution was performed and a 5-ml volume was inoculated in 495 ml of HI broth. A growth curve was established for this strain by measuring $OD_{610\,nm}$ and plate count on HIA. Total counts were carried out using 4′,6-diamidino-2-phenylindole (DAPI) staining (Porter and Feig, 1980). All measurements were made hourly over a 3 h period.

DNA extracted from the clinical strain of *V. parahaemolyticus* Vp101 (Centre National de Référence des Vibrions et du Choléra, CNRVC, Paris, France) served as positive control for PCR.

Induction of the VBNC state

VBNC cells were obtained using the method described elsewhere (Coutard *et al*., 2005). Briefly, a 200-ml volume of the culture in mid-logarithmic phase ($OD_{610\,nm} \approx 0.2$) was centrifuged at 2500 *g* for 10 min. The cell pellet was washed twice in 200 ml artificial seawater (ASW) at 10°C. Washed cells were inoculated in a final 2-l ASW volume in a 5-l flask and incubated at 4°C, with shaking (70 r.p.m.), in the dark. Plate counts and total counts were determined daily using 2% NaCl HIA and incubation at 37°C for 24 h and by DAPI staining, respectively. The VBNC state was reached when a count of one, or less than one, culturable cell in 20 ml of ASW was observed. The experiments were carried out in duplicate.

Recovery of culturability by temperature upshift

To investigate the processes involved in recovery of culturability, a series of two independent experiments was performed. These experiments were carried out employing two ASW microcosms containing VBNC cells of *V. parahaemolyticus* Vp5 sampled at mid-logarithmic phase and in two separate and different cultures. For each experiment, aliquots of 0.1, 1 and 10 ml were sampled at days 1, 4, and 7 after entry into the VBNC state. These aliquots were incubated in flasks at 20 and 37°C, with shaking (70 r.p.m.), in the dark. Plate counts (on 2% NaCl HIA) and total counts (DAPI) were performed, as described previously, on days 1, 2, 6, 10, 20 and 41, after temperature upshift.

Confirmation by PCR

To confirm identification of colonies observed before and after temperature upshift, a colony was picked and the cells washed three times with 1 ml of buffered physiological saline and centrifuged at 2500 *g* for 5 min at room temperature. The pellet

was suspended in 50 μl of sterile deionized water. After heating at 100°C for 15 min, an aqueous phase, containing nucleic acid, was obtained by centrifugation at 9200 *g* for 15 min. Nucleic acid was quantified by spectrophotometry at 260 nm and stored at –80°C until use. For detection of the *tdh2* gene in *V. parahaemolyticus* Vp5, primers were designed from *V. parahaemolyticus* RIMD 2210633: F-tdh2 (5′-CAACTTTTAATACCAATGCAC-3′, forward primer) and R-tdh2 (5′-AATAGAATCTTCATCTTCACC-3′, reverse primer). PCR was performed in a 40-μl mixture containing 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostics, Meylan, France), 200 μM of each deoxyribonucleotide triphosphate, 1 U of *Taq* DNA polymerase (Roche Diagnostics), 500 nM of each primer and 100 ng of DNA. Negative and positive controls were prepared using deionized water and the DNA was extracted from *V. parahaemolyticus* Vp101. The PCR program was conducted in a Peltier Thermal Cycler-200 (MJ Research Inc., Waltham, MA, USA) as follows: 94°C for 5 min; 35 cycles of an initial denaturation at 94°C for 30 s; annealing at 58°C for 30 s and extension at 72°C for 45 s; and a final extension at 72°C for 7 min. 15 μl of the reaction mixture was resolved with 2.5 μl of gel loading solution (Sigma Aldrich Chimie, St Quentin Fallavier, France) by electrophoresis in 1.5% agarose gel (Eurobio, Les Ulis, France).

Regrowth of V. parahaemolyticus *in ASW*
Cells that had been VBNC in ASW microcosms for 1 month were centrifuged for 4 h in the dark. The ASW microcosm supernatant was filtered through a 0.22-μm pore size filter (Nalge Europe Ltd, Neerijse, Belgium) to remove intact cells $>0.22\,\mu$m before inoculation with culturable cells. Two independent experiments were performed. Each experiment comprised four microcosms containing 250 ml of ASW microcosm supernatant filtered previously and four microcosms containing 250 ml of filtered and autoclaved ASW. The four microcosms of each series were inoculated at final concentrations of 0 (negative control), 0.1, 1 and 10 colony-forming unit (CFU) ml^{-1}. For each experiment, the eight microcosms were stored at room temperature with shaking (70 r.p.m.), in the dark. Plate counts were performed at days 0, 1, 2, 3, 5 and 7 on 2% NaCl HIA, in triplicate. Generation times (t_g) were calculated using the following equation:

$$t_g = \ln(2)/(V \ln(10))$$

where V signifies evolution of the decimal logarithm of the cell population, with respect to time. ln(2) represents exponential growth of the bacteria.

Scanning electron microscopy
Cells in VBNC state for 1 day (2 ml from the ASW microcosm at 4°C) were fixed with 2% (vol/vol) glutaraldehyde for 4 h. The fixed cells were capillary filtered, using 0.2-μm pore size tissue culture inserts (NunC, Roskilde, Denmark) and dehydrated by successive ethanol washing (from 70 to 100%), after which a critical point was reached. A 10-ml volume of cells, after temperature upshift, was fixed with 2% (vol/vol) glutaraldehyde and filtered through 0.2-μm pore size polycarbonate black filters (Dominique Dutscher S.A., Brumath, France). The gold-coated samples were examined by scanning electron microscopy (SEM) (Philips XL 30 LaB6, Philips Optique Electronique, Limeil Brevannes, France).

Transmission electron microscopy
Two days after temperature upshift at 20°C, 200 ml of the VBNC cells were fixed with 2% (vol/vol) glutaraldehyde for 24 h at room temperature, centrifuged at 2500 *g* for 10 min and the cell pellet suspended in cacodylate buffer and post-fixed, using 1% osmium tetraoxide for 1 h. Cells were centrifuged at 2500 *g* for 5 min and washed in 0.1 M Sorensen's phosphate buffer at pH 7.4. After centrifugation at 2500 *g* for 5 min, the cells were dehydrated in successive ethanol washes (30–100%). Each step was followed by centrifugation at 2500 *g* for 5 min. Two 15 min washes with propylene oxide were carried out and preparations embedded in a mixture (vol/vol) of propylene oxide and EMbed 812-Araldite 506 resin (Euromedex, Souffelweyersheim, France) for 1 h. The mixture was removed and replaced by fresh, pure resin. Resin polymerization was performed for 24 h at 60°C. Samples for transmission electron microscopy (TEM) were prepared using an ultramicrotome (Reichert-Jung Ultracut E, Leica, Vienna, Austria). Sections with a 50-nm thickness were stained using 3.5% uranyl acetate and 0.4% lead citrate and were observed with a JEM-1010 transmission electron microscope (JEOL Europe SAS, Croissy-sur-Seine, France).

Microanalysis
An energy-dispersive spectroscopic analysis of chemical elements in the bacteria and the 'bud' or asymmetric daughter cell was performed by X-ray energy-dispersive analysis (EDAX). Samples previously fixed in 2% (vol/vol) glutaraldehyde were washed in water, centrifuged for 5 min at 2500 *g* and deposited on 99.98% pure nickel foil (Goodfellow, Lille, France). The samples were coated with gold particles. The electron beam was focused at the location where elemental composition was to be determined. The data are reported in weight percentage of each chemical element observed.

Results

Recovery of culturability of VBNC cells after temperature upshift
A decrease in culturability was observed for ASW microcosms incubated at 4°C, and the VBNC state

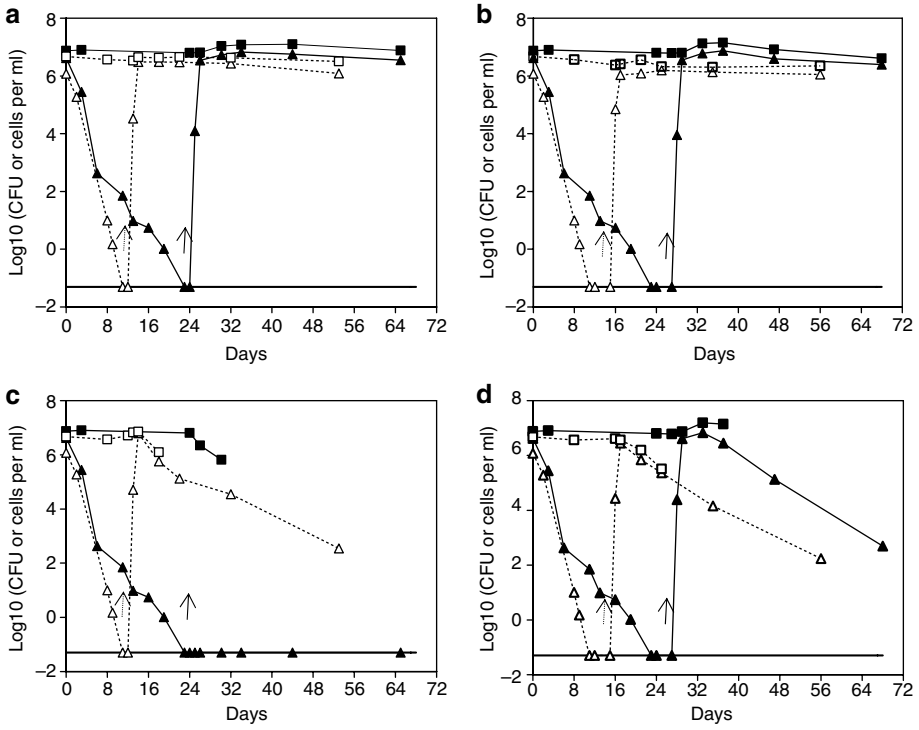

Figure 1 Recovery of culturability by VBNC cells. Temperature was increased to 20°C (**a** and **b**) and 37°C (**c** and **d**) 1 day (**a** and **c**) or 4 days (**b** and **d**) after the VBNC state was reached. Results of two independent experiments are shown (dotted and solid lines). Arrows indicate temperature upshift. Plate counts (triangle) were determined using 2% NaCl HIA at days 1, 2, 6, 10, 20 and 41 after temperature upshift. Total counts (square) were determined by DAPI staining. Detection limit of CFU was 0.05 CFU ml^{-1} (—).

was reached at days 11 and 23 in the two experiments (Figure 1). Recovery of culturability at 20 and 37°C was not observed for the 0.1 and 1-ml aliquots (data not shown). In the first experiment, only the 10-ml aliquots showed increases in the number of CFU at 20°C after 1 and 4 days in the VBNC state and at 37°C after 4 days in the VBNC state (Figure 1a, b and d, respectively). However, no recovery of culturability was observed in the sample incubated at 37°C after 1 day of VBNC state (Figure 1c). In the second experiment, recovery of culturability was observed in each of the 10-ml aliquots at 20 and 37°C, after 1 and 4 days in the VBNC state (Figure 1). No recovery was observed for VBNC cells at 7 days in the two experiments (data not shown). These results suggest that a subpopulation of VBNC cells could be cultured after temperature upshift and that the potential of culturability of the total population changes with time. The generation times of cells that did grow were 1.34, 1.37 and 1.27 h for the first experiment, and 1.24, 1.17 and 1.26 h for the second experiment, calculated after temperature upshift at 20°C for 1 day and 4 days in the VBNC state and at 37°C after 4 days in the VBNC state, respectively (Figure 1a, b and d). No significant difference between the generation times obtained in the two independent experiments was observed ($P > 0.05$). A decrease in culturability was observed after 6 and 41 days, following temperature increase at 37°C. This reduction was not observed for VBNC cells incubated at 20°C.

PCR identification of V. parahaemolyticus *Vp5*

Colonies appearing on agar were of various sizes when compared at day 0 in ASW at 4°C (Figure 2a) with day 6 after temperature upshift at 20 or 37°C (Figure 2b and c, respectively). To rule out contamination, *V. parahaemolyticus*-specific PCR, targeting the *tdh2* gene, was used to test colonies on the plates incubated at 20 and 37°C. The amplicons obtained confirmed the cultures to be *V. parahaemolyticus* (data not shown).

Heterogeneity of the VBNC population

VBNC cells were coccoid shaped, with a diameter of 1.1–1.3 μm (Figure 3a and b). After dehydration and critical point drying, cells of the same size, that is between 0.8 and 1.2 μm diameter (Figure 3c and d) were observed, along with larger, flattened cells (c. 2 μm in diameter), appearing to lack internal cell contents (Figure 3c and d). Cells in the size range of 0.8–1.2 μm appeared to present more or less normal structural integrity, representing 5–10 per 100 cells whereas the larger, flattened cells appeared to have lost their cytoplasmic contents.

Phenotypic characterization of cells after recovery of culturability

From results of the above experiments, regrowth of *V. parahaemolyticus* Vp5 from the VBNC state was concluded to occur after temperature upshift to 20 and 37°C. By SEM, cell division was observed (Figure 4a and b), with binary fission yielding daughter cells (Figure 4c) during recovery after temperature upshift at 20 or 37°C. Large, flattened cells were always observed (Figure 4g). In addition, an interesting phenomenon was also noted, namely formation of 'budding' or very small coccoid cells on the surface of some of the cells (Figure 4d, e, f, h and i) and appearance of asymmetric cell (Figure 4g, h and i). These unusual observations were confirmed by TEM, which revealed both symmetric (Figure 5a and b) and asymmetric cell division (Figure 5c and d), with septum formation in both cases. Inner and outer membranes were also observed, as well as cell cytosol in all cases (Figure 5).

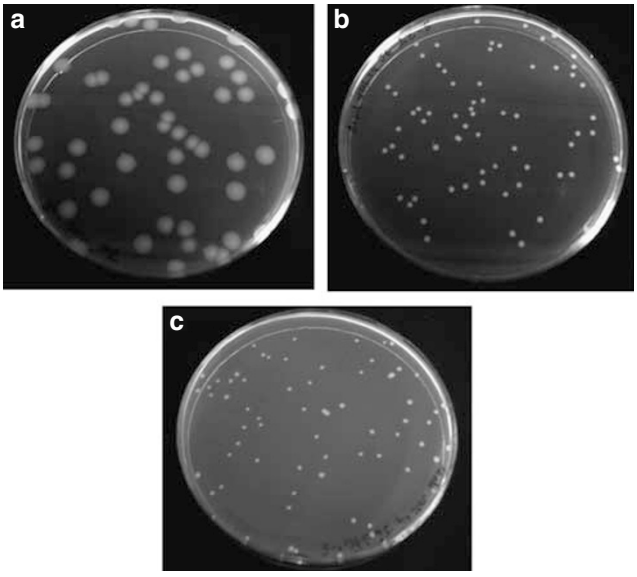

Figure 2 Variations in colony morphology of *V parahaemolyticus* after temperature upshift. Plate counts of *V. parahaemolyticus* Vp5 in ASW samples (10 ml) at 4°C (day 0) (**a**) and after a temperature increase to 20°C (**b**) or 37°C (**c**) for 6 days. Temperature upshift was determined 4 days after the VBNC state was achieved. The HIA 2% NaCl plates were incubated at 37°C for 24 h.

Element composition of both symmetrical and asymmetrical division in V. parahaemolyticus

Results of EDAX showed that the product of asymmetrical cell division has similar elemental composition (Figure 6). The main elements detected were carbon, nitrogen and oxygen, in percentages of 14.14, 9.52 and 3.02, respectively, in the mother cell and 15.31, 7.90 and 3.10 in the asymmetric daughter cell (Figure 6). No difference in composition was observed by this analysis.

Recovery of culturability of V. parahaemolyticus *Vp5 in ASW*

A generation time was calculated for culturable cells incubated in ASW at 20°C, with or without nutrient

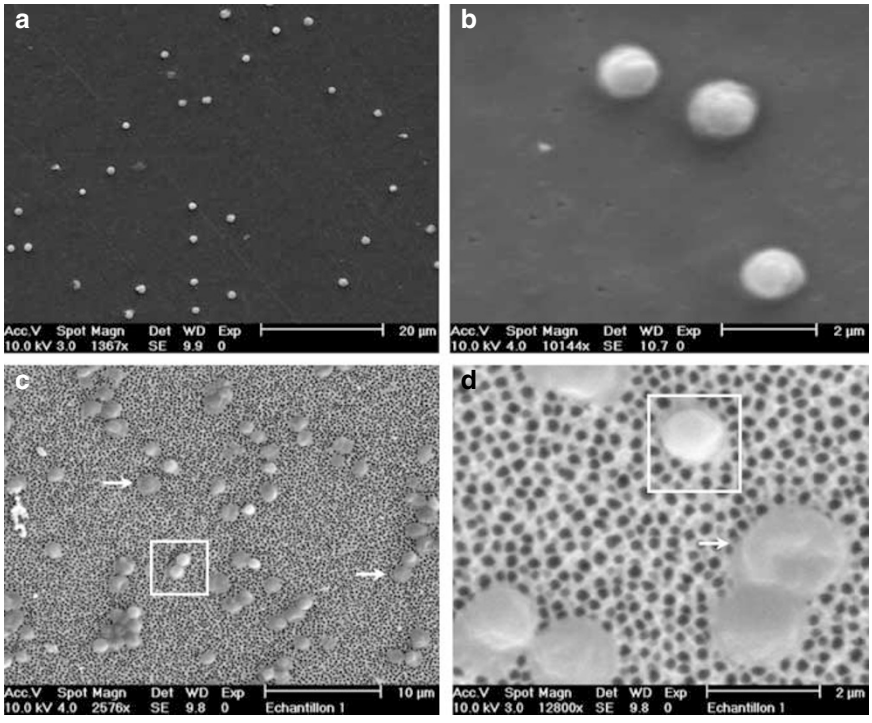

Figure 3 Morphological heterogeneity of the VBNC population. VBNC *V. parahaemolyticus* Vp5 in ASW at 4°C after 1 day. Samples were treated without (**a** and **b**) or with (**c** and **d**) dehydration and critical point drying and observed using SEM. Arrows indicate flattened or empty cells and squares indicate intact cells.

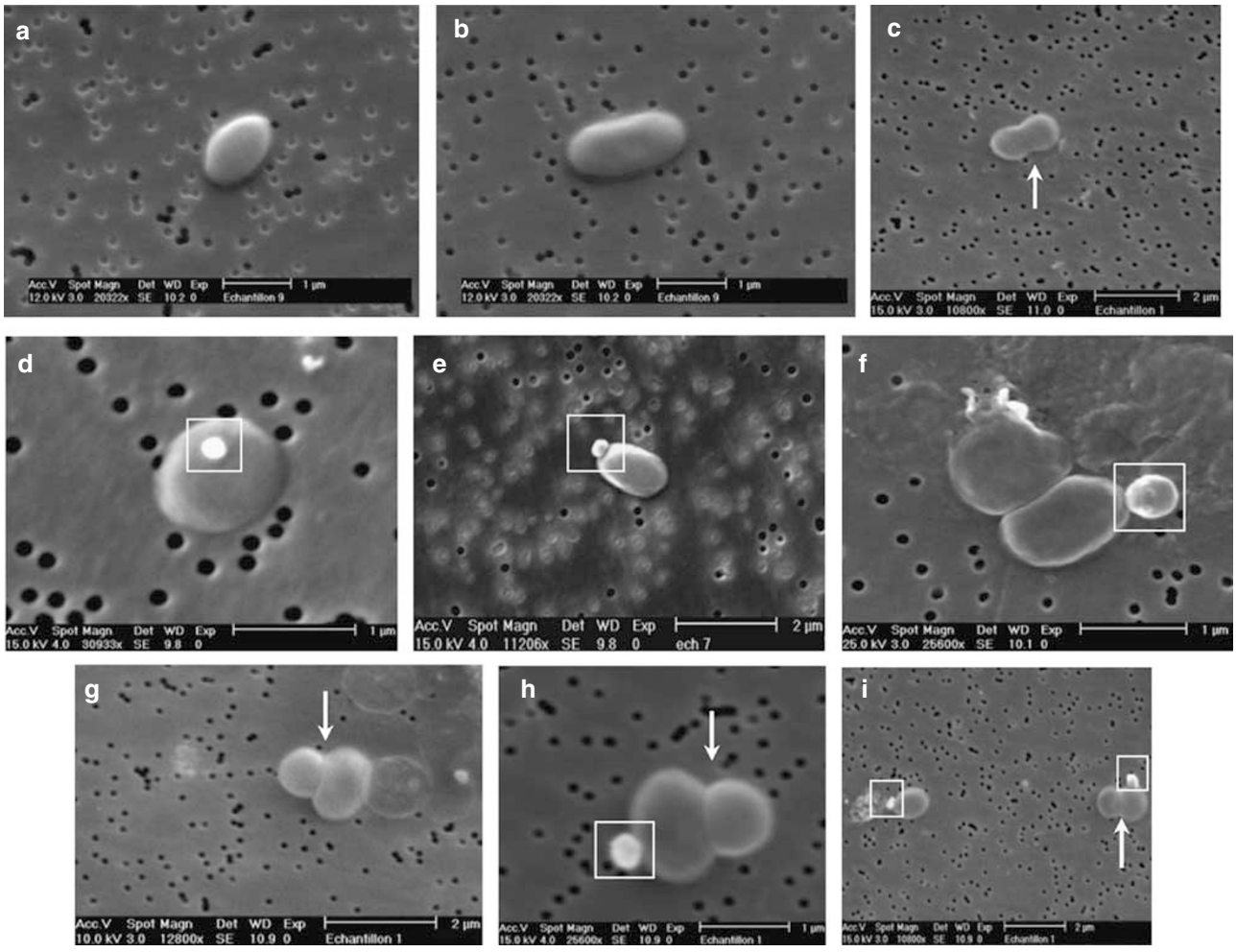

Figure 4 Morphological analysis using SEM of VBNC *V. parahaemolyticus* after temperature upshift. The day after the VNBC state was achieved (day 23), the temperature was increased to 20°C and SEM was performed the next day. Squares show formation of small coccoid cells (**d**, **e**, **f**). Arrows show traditional cell division (**c**) or asymmetric division (**g**, **h**, **i**) or 'budding'.

added to the filtrate. During the first 24 h of growth, cells incubated in 0.22 μm filtered–autoclaved ASW microcosms at concentrations of 0.1, 1 and 10 CFU ml^{-1} had generation times of 3.61, 3.54 and 3.64 h, respectively. The maximum number of culturable cells were 2×10^5, 5×10^5 and 7×10^5 CFU ml^{-1}, respectively, and was reached at 3–5 days (Figure 7a, b and c). For the same cell concentrations, the generation times were 1.65, 1.72 and 1.45 h in the ASW microcosms to which filtrate from the VBNC microcosms had been added. The initial total count was 8.2×10^6 ml^{-1} and from 0.1, 1 and 10 CFU ml^{-1} microcosms, 4.1×10^6, 4.5×10^6 and 3.5×10^6 CFU ml^{-1}, respectively (Figure 7a, b and c, respectively). Without culturable cells derived from growth in ASW, the counts were 47.6, 48.8 and 34.2 culturable cells per 100 cells in the VBNC filtrate (Figure 7a, b and c, respectively).

Discussion

There is a debate whether those culturable cells that are recovered after temperature upshift result from resuscitation of all cells of the initial inoculum that had become VBNC, from regrowth of only a few VBNC cells remaining viable (able to revert to active growth), or simply growth of a very few undetected culturable cells. In the last mentioned case, the cell fraction able to grow would comprise stressed cells (perhaps oxidative stress), that is, cells degenerating toward death, according to Bogosian *et al.* (2000) and Mizunoe *et al.* (2000). If that was the case, the VBNC state would not represent an adaptive programming of cells, like spore formation resulting from differentiating cells, but rather a transitory physiological state before cell death. Some investigators maintain that resuscitation represents a reversion of the metabolic and physiological processes in VBNC cells, without growth (Nilsson *et al.*, 1991; Oliver *et al.*, 1995; Whitesides and Oliver, 1997), whereas many others suggest that it represents resumption of growth of VBNC cells (Weichart *et al.*, 1992; Firth *et al.*, 1994).

Demonstration that a large portion of a population of cells lose their cell content eliminates resuscitation as an explanation for recovery of culturability

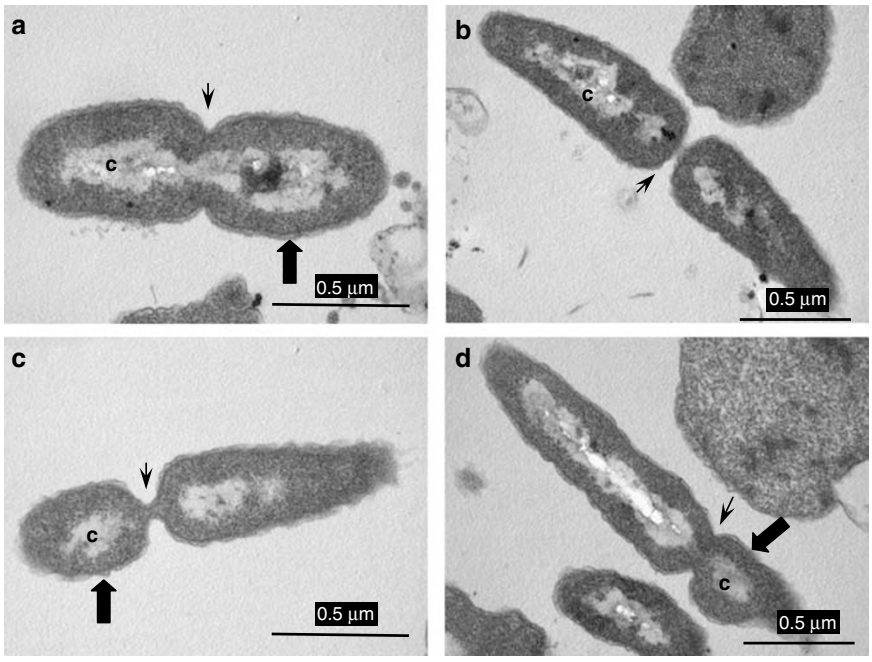

Figure 5 Morphological analysis using TEM of VBNC *V. parahaemolyticus* after temperature upshift. One day after the VNBC state of *V. parahaemolyticus* Vp5 was achieved, the temperature was increased to 20°C. TEM was performed after 48 h. Small arrows show traditional cell division (**a** and **b**) or asymmetric division (**c** and **d**) with septum formation. Large arrows indicate the presence of outer and inner membranes while C indicates cell cytosol.

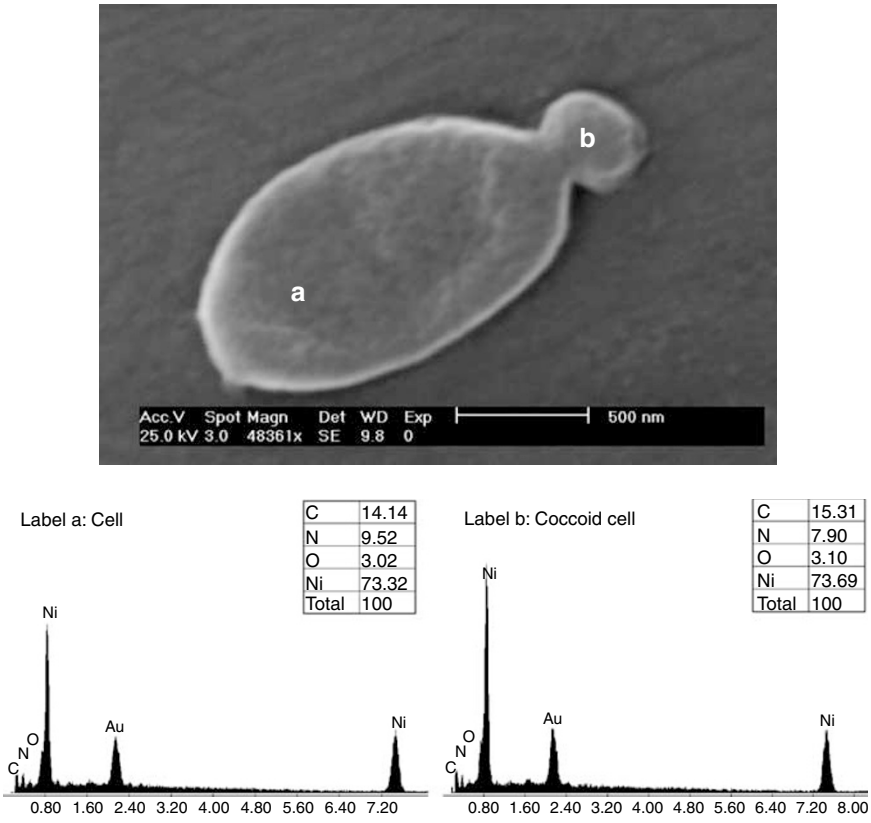

Figure 6 Identification of chemical elements. A comparison of the atomic structure of the ovoid cell (**a**) and the coccoid cell (**b**) was made after temperature upshift (20°C for 48 h) and 24 h after the VBNC state had been reached. Chemical elements detected included: C, carbon; N, nitrogen; O, oxygen; Ni, nickel and Au, gold. The table inserts indicate weight percentage of each element, except gold.

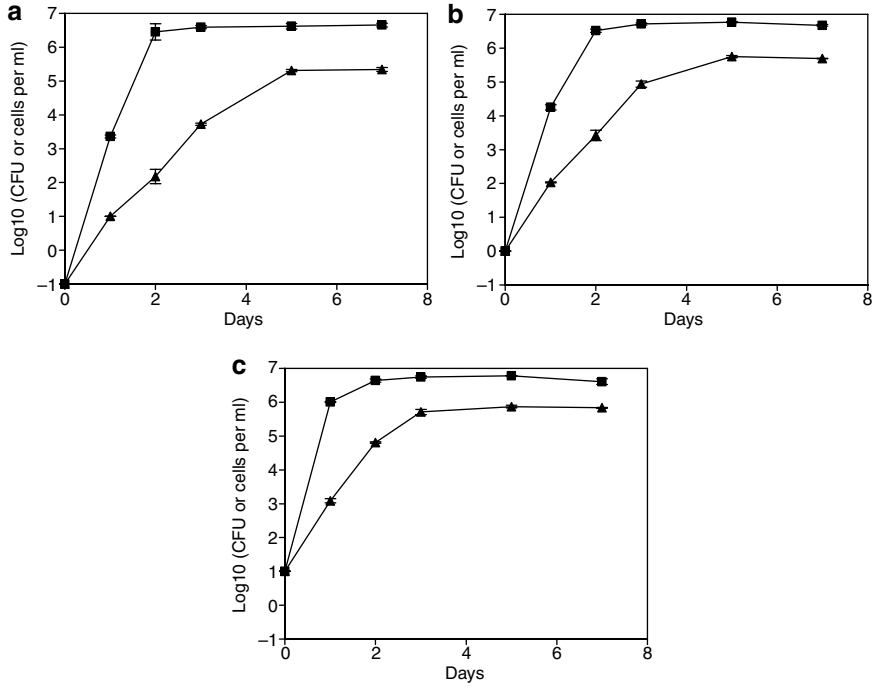

Figure 7 Growth of culturable *V. parahaemolyticus* in ASW. Growth of *V. parahaemolyticus* Vp5 was monitored in filtered–autoclaved ASW (triangle) or in ASW with nutrient from dead cells (square) at 20°C. The ASW microcosms were inoculated at final concentrations of 0.1 (**a**), 1 (**b**) and 10 (**c**) CFU ml^{-1}. The data are expressed as the means ± s.d.'s ($n = 2$).

after temperature upshift. If only some of the VBNC cells, representing only a portion of the total VBNC population (a sub-population), return to approximately the same population size as the initial number of culturable cells, this could not result from simple reversal of metabolic and physiological processes. Cell division, or a mechanism like exsporulation, is involved in the appearance of culturable bacteria from the VBNC population. Results obtained in previous studies with *V. vulnificus* (Nilsson *et al.*, 1991; Oliver *et al.*, 1995; Whitesides and Oliver, 1997) and *V. parahaemolyticus* (Bates and Oliver, 2004; Wong *et al.*, 2004) have been interpreted as resuscitation of the entire VBNC population. However, in the study reported here, the decrease in culturability of *V. parahaemolyticus* Vp5 was noted after a temperature downshift to 4°C, with recovery of culturability of VBNC cells after temperature upshift occurring only for a relatively large volume (10 ml) of the VBNC population. This observation suggests that either only a fraction of the cell population is able to recover culturability or a sufficiently large number of VBNC cells are needed for recovery of culturability (for example signalling phenomenon) and that physiological heterogeneity exists within the cell population. These results are similar to those obtained by Weichart *et al.* (1992) and Jiang and Chai (1996), who reported recovery of VBNC cells of *V. vulnificus* and *V. parahaemolyticus* arising from regrowth of few cells, but not resuscitation of all VBNC cells. In the experiments reported here, a given volume of inoculum was necessary for recovery of culturability after temperature upshift, contrary to the observations of Whitesides and Oliver (1997) for *V. vulnificus*. SEM and TEM observation confirmed the heterogeneity of the VBNC population. Furthermore, all cells did not react identically to the temperature shifts. Some cells remained intact, whereas others did not.

Weichart *et al.* (1997) concluded that only a fraction of VBNC *V. vulnificus* retained integrity of their nucleic acids (DNA and RNA). Our results indicate that only a sub-population of VBNC *V. parahaemolyticus* was able to recover culturability immediately after the temperature upshift. If the heterogeneity of the *V. parahaemolyticus* population is related to the emergence of stress-induced mutations in the DNA that participates in adaptative evolution of the cells, such as has been described for *E. coli* (Bjedov *et al.*, 2003), then a protection (σ^S system) and repair (SOS system) could participate in the survival of some of the cells and in generation of genetic alteration (Matic *et al.*, 2004). In that case, a few phenotypes survive, representing the VBNC state constituting those cells able to induce a survival mechanism. The initial cells would not have the intrinsic capacity to enter the VBNC state, but some could acquire this property when subjected to stress. Recovery of culturability was reached 48 h after temperature upshift. The hypothesis that VBNC recovery derived from growth of only a few cells that remained culturable, but not detected, is unrealistic, as the limit of detection in

the study was less than 1 CFU per 20 ml. Furthermore, when incubated at 20°C in an oligotrophic medium, *V. parahaemolyticus* reached population sizes from inocula of 0.1, 1 and 10 CFU ml^{-1} of 2×10^5, 5×10^5 and 7×10^5 CFU ml^{-1} within 5 days, respectively. These results are very similar to those obtained by Ravel *et al.* (1995) for *Vibrio cholerae*, with significant growth observed at 30°C in ASW, from 10^2 to 6.2×10^5 CFU ml^{-1} in 24 h.

Considering that Postgate and Hunter (1962) calculated that 50 dead cells of *E. coli* were necessary to support division of a single cell, cryptic growth cannot explain the increase in the number of cells observed in this study. Results for *V. parahaemolyticus* Vp5 showed that ASW with nutrients provided by the filtrate from the VBNC microcosm supported regrowth of a few viable cells. That is, 100 VBNC cells in the original microcosm provided nutrients that made it possible to have 34.2–48.8 culturable cells, with generation times of 1.45–1.72 h, representing generation times approximately twice that in filtered–autoclaved ASW. The generation times observed after temperature upshifts at 20 and 37°C were similar to those obtained under conditions of cryptic growth, supporting the hypothesis that cell division is involved in the temperature-dependent recovery of VBNC *V. parahaemolyticus*. These findings are in agreement with those of Weichart *et al.* (1992) for *V. vulnificus*, who concluded that dead cells could provide sufficient nutrient to restore up to 58% of the initial culturable count. The microscopic observations support regrowth of cells, with evidence of cell division. Oliver (1993) defined resuscitation as 'a reversal of the metabolic and physiologic processes that resulted in nonculturability, resulting in the ability of the cells to be culturable on those media normally supporting growth of the cell'. The results reported here suggest that the VBNC cells return from their state of survival, adaptation or dormancy, by initiating regrowth, rather than simply recommencing metabolic functioning of the original cell population.

SEM and TEM results support the hypothesis that recovery of culturability is not simply recovery of binary fission, but reveal atypical mechanisms for bacterial division. The coccoid cells showed nearly identical atomic composition as the mother cells, namely organic composition and appear as the daughter cells, with an outer and inner membrane, as well as cytoplasmic ultrastructure. Very small, coccoid cells can reach very large population sizes in an environment of limited nutrient, as suggested by Jiang and Chai (1996). A mechanism for coccoid cell formation may, in fact, represent an adaptation of *V. parahaemolyticus* and other species of the family *Vibrionaceae*, to constantly changing environmental conditions; for example, that which is encountered in an estuary throughout a full annual climate (seasonal) cycle. More importantly, this phenomenon demonstrated by *V. parahaemolyticus* must also be considered in terms of public health risk. Expression of housekeeping and virulence genes during regrowth from the VBNC state would be a fruitful study to undertake.

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