RESEARCH ARTICLE

Inability of *Escherichia coli* to resuscitate from the viable but nonculturable state

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Abstract

After induction of the viable but nonculturable (VBNC) state in *Escherichia coli* populations, we analysed abiotic and biotic factors suggested to promote the resuscitation process. The response to the stressing conditions implied the formation of three subpopulations, culturable, VBNC and nonviable. In most adverse situations studied, the VBNC subpopulation did not represent the dominant fraction, decreasing with time. This suggests that, in most cases, the VBNC is not a successful phenotype. Combining methods of dilution and inhibition of remaining culturable cells, we designed a working protocol in order to distinguish unequivocally between regrowth and resuscitation. Reversion of abiotic factors inducing nonculturability as well as prevention of additional oxidative stress did not provoke resuscitation. Participation of biotic factors was studied by addition of supernatants from different origin without positive results. These results indicate that the *E. coli* strain used is not able to resuscitate from the VBNC state. VBNC cells release into the surrounding medium, and could thus aid in the survival of persisting culturable cells. The formation of a VBNC subpopulation could thus be considered as an adaptive process, designed for the benefit of the population as a whole.

Introduction

Since Xu *et al.* (1982) defined the phenomenon referred to as the 'viable but nonculturable' (VBNC) state, the number of species described as entering it is constantly increasing (Oliver, 2005). The list includes nonpathogens as well as a large number of human pathogens. The term VBNC cells was coined for those bacterial cells with detectable metabolic function, but not culturable by available methods (Roszak & Colwell, 1987). This raises serious questions regarding, for example, the interpretation of routine water or food testing in which it is assumed that infecting bacteria can be detected by their ability to grow in culture.

The VBNC state has been extensively studied and there is abundant information about the physiology, biochemistry and genetics of cells as they are entering this state (see review by Oliver, 2005). Thus, it has been reported that VBNC cells exhibit reduction in nutrient transport, respiration rates and macromolecular synthesis, but that novel stress proteins (Morton & Oliver, 1994; Srinivasan *et al.*, 1998) are formed and cytoplasmic membrane fatty acid composition (Day & Oliver, 2004) and cell-wall peptidoglycan (Signoretto *et al.*, 2000, 2002) are modified. Moreover, gene expression has been demonstrated (Lleó *et al.*, 2000; Smith & Oliver, 2006). However, there is ongoing intense debate regarding the biological significance of this phenomenon.

Different models have been proposed to explain this phenomenon. Some authors have hypothesized that cells exposed to hostile environments become nonculturable due to cellular deterioration, which can be a stochastic (Bogosian & Bourneuf, 2001) or genetically programmed (Nyström, 2003; Aertsen & Michiels, 2004) phenomenon. This might be an adaptive strategy ensuring the persistence of the population. Cells with reduced or no colony-forming capacity could provide nutrients for remaining culturable cells and allow the presence of persistors under adverse conditions (Lewis, 2000; Cuny *et al.*, 2005).

Another theory suggests that nonculturability is the culmination of an adaptive pathway generating dormant survival forms, similar to spore formation in differentiating

bacteria (McDougald *et al.*, 1999; Kell & Young, 2000). The VBNC state could be a significant means of survival if the VBNC cells were able to become culturable again, i.e. cells underwent a process of resuscitation (McDougald *et al.*, 1999; Oliver, 2005; Keep *et al.*, 2006a).

Demonstrating resuscitation is not always a simple matter. A major problem is the difficulty in showing conclusively that the 'resuscitated' population comes from a true resuscitation and not the result of the growth of culturable cells hidden in the nonculturable population (Bogosian *et al.*, 1998; Kell *et al.*, 1998; Bogosian & Bourneuf, 2001). On the other hand, resuscitation may be an extremely intricate event and complex conditions or factors must sometimes be employed. Yamamoto (2000) classifies the resuscitation factors into three, nonexclusive categories.

The first category refers to the removal of environmental stress (by addition of nutrients to starved cells, change to a suitable temperature, return to darkness, etc.) (Magariños *et al.*, 1997; Whitesides & Oliver, 1997; Lleó *et al.*, 2001; Ohtomo & Saito, 2001; Ramaiah *et al.*, 2002; Gupte *et al.*, 2003). Thus, Whitesides & Oliver (1997) and Wong *et al.* (2004) have described that the VBNC state in *Vibrio* is typically resuscitated by temperature upshift treatment; and Magariños *et al.* (1997) have obtained similar results in *Pasteurella pisticida* when nutrient conditions are favourable.

A second group of factors includes the repairing of damage and the activation of replicative functions, as described by Wai *et al.* (1996) and Kurokawa *et al.* (1999), who recovered culturable *Vibrio vulnificus* or *Helicobacter pylori* cells by heat-shock in cultures grown on medium containing ammonium salts.

Finally, signal molecules, which are agents to stimulation and regulation of physiological conditions, are also considered. Bacterial cells require the presence of adjoining cells (or of their products) in certain conditions for their growth and survival (Mukamolova *et al.*, 1998; Srinivasan *et al.*, 1998; Kell & Young, 2000). Kaprelyants *et al.* (1994), Mukamolova *et al.* (1998), Mukamolova & Kormer (1999) and Panutdaporn *et al.* (2006) have described resuscitation of VBNC cells after exposure to cell-free supernatants obtained from cultures growing under optimal conditions and, thus, compounds secreted and excreted by the population during growth are included.

The aim of the present investigation was to study the influence of abiotic and biotic factors on the resuscitation of VBNC populations of *E. coli*. Thus, the ability to resume culturability is tested on returning the conditions inducing the loss of culturability and entry to the VBNC state as well as by addition of supernatants obtained from different growth phases of *E. coli*, *Pseudomonas fluorescens* and *Enterococcus faecalis* cultures.

Materials and methods

Bacterial strains

Three bacterial strains were used in this study. *Escherichia coli* STCC 416 (Spanish Type Culture Collection) was maintained under adverse conditions to provide cellular suspensions in VBNC state. This *E. coli* strain as well as *P. fluorescens* CHA0 (Hase *et al.*, 1999) and *Enterococcus faecalis* pMV158GFP (Nieto & Espinosa, 2003) were grown to obtain supernatants. The *E. coli* strain was maintained on nutrient agar (Oxoid), *P. fluorescens* CHA0 on King's B agar (Oxoid) and *Enterococcus faecalis* on brain heart agar (Merck) at 4 °C. The *E. coli* strain was examined for susceptibility to antimicrobial drugs. Minimal inhibitory concentrations (MICs) were determined as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997).

Bacterial counts

For direct determinations, bacteria were retained on 0.22 μm pore-size black polycarbonate filters (Millipore) and examined via epifluorescence microscopy. The total number of bacteria (TDC) was determined by means of the standard acridine orange direct procedure (Hobbie *et al.*, 1977).

Viable bacteria were estimated as bacteria with intact cytoplasmic membranes ($MEMB^+$). These $MEMB^+$ bacteria were counted with the aid of the Live/Dead® BacLight™ kit (Molecular Probes) as described by Joux *et al.* (1997). Those bacteria with intact cytoplasmic membranes (green fluorescence, $MEMB^+$) and the permeabilized bacteria (red fluorescence) were enumerated separately.

Culturability, expressed as CFUs, was evaluated by incubating the *E. coli* cells on tryptone soy agar (TSA) and TSA supplemented with catalase (11 U mL^{-1}) for 24 h at 37 °C. There were no significant differences between these counts (data not shown).

Percentages of each cellular subpopulation were calculated with respect to TDC counts: culturable cells (C) directly from CFU, nonculturable cells (NC) as TDC–CFU, VBNC as $MEMB^+$–CFU and nonviable cells (NV) as TDC–$MEMB^+$.

Preparation of nonculturable *E. coli* suspensions

Suspensions of nonculturable cells consist of VBNC (cells that maintain intact cytoplasmic membranes) and NV (cells that retain their morphology but do not maintain intact cytoplasmic membranes) cells. To obtain these suspensions, *E. coli* populations were exposed to adverse conditions: starvation in sterile saline solution (0.9% NaCl, SS) in darkness or under exposure to visible radiation; starvation

in seawater and exposure to acid environment and to hydrogen peroxide.

In the inocula preparation, *E. coli* STCC 416 was cultured aerobically in M9 minimal broth (MB) supplemented with 0.2% glucose (MBG) with shaking (120 r.p.m.) at 37 °C. Cells from the exponential growth phase were harvested by centrifugation (4330 ***g*** for 10 min) and washed three times in sterile SS. Finally, the pellet was resuspended in sterile SS.

The assays were carried out in Erlenmeyer flasks containing 2 L sterile SS or sterile natural seawater (SW). So that organic residues might be avoided, glass flasks were cleaned in acid beforehand, rinsed with deionized water, and kept at 250 °C for 24 h. Flasks were inoculated to a final density of 10^7 cells mL^{-1}. Some SS flasks were incubated at 20 or 37 °C with shaking (160 r.p.m.) in darkness or under illumination (five lamps; Philips TLD 30W/54); SW flasks were incubated at 20 or 37 °C in darkness. In all experiments, periodically, samples were taken in triplicate for monitoring the formation of nonculturable cells.

An acid environment was achieved in SS flasks by adding HCl to adjust the pH to 2.5–3 and oxidation was provoked by exposure to 0.2% hydrogen peroxide. These flasks were incubated for 15 min at 20 °C. Before bacterial counts, pH was neutralized by addition of NaOH (1 M) and H_2O_2 removed by addition of catalase (11 U mL^{-1}).

When culturability was < 0.1 CFU mL^{-1}, cells were harvested by centrifugation (4330 ***g*** for 10 min) and filtration (0.22 μm pore-size GVWP membrane, Millipore) and resuspended in sterile SS. When culturability remained higher than 0.1 CFU mL^{-1}, nonculturable cells were collected using a discontinuous gradient of Urografin (Urografin 76%, Shering Alman) (Siegele *et al.*, 1993). One millilitre of cellular suspension was layered on the top of the gradient and centrifuged for 14 h at 140 700 ***g*** at 4 °C in a swinging bucket rotor (Centrikon T-2190, rotor TST 28.38, Kontron Inst). When centrifugation was finished, nonculturable cells were concentrated into the lower band. These cells were collected and resuspended in sterile SS.

Preparation of supernatants

Previously, growth curves of the three bacterial strains were made. Culture media were elaborated with different carbon sources as well as with increasing concentrations of nutrients (data not shown). Flasks containing 2 L of different media were inoculated with *c.* 10^4 cells mL^{-1}. *Escherichia coli* and *Enterococcus faecalis* were cultured at 37 °C and *P. fluorescens* at 26 °C with shaking (120 r.p.m.). Culture media that supported a high rate of growth and maximal increase in the bacterial densities from the lowest nutrient concentrations were selected (Arana *et al.*, 2004). This selection was made in order to minimize the effect of nutrient addition (abiotic factor) on the resuscitation processes. Finally, *E. coli* and *Enterococcus faecalis* were cultured aerobically in MB supplemented with 0.05% glucose and with 0.03% yeast extract (MBGYE) and *P. fluorescens* in 20% diluted Luria–Bertani broth (DLB).

During the growth of the three microorganisms, supernatants (SN) of the middle exponential growth phase (density of 10^6–10^7 cells mL^{-1}) and of the early stationary growth phase (density of 10^9 cells mL^{-1}) were obtained by centrifugation (4330 ***g*** at 4 °C for 10 min) and filtration through sterile GVWP membrane filters (0.22 μm). Supernatants were kept at − 20 °C in glass bottles without organic matter.

Filtered supernatants were incubated with shaking (120 r.p.m.) at 37 and 26 °C to test the growth of cells which might potentially pass through filters. Moreover, to test the ability of supernatants to support *E. coli* growth, cells from the early stationary phase of growth were inoculated into different supernatants and cells were periodically enumerated.

pH and dissolved monomeric carbohydrates (DMCH) were characterized in the supernatants. DMCH were measured by means of the 3-methyl-2-benzothiazolinone hydrazone hydrochloride assay (Jonhson & Sieburth, 1977), with a Synergy™ HT multidetection microplate reader (Biotek). All values are given here as glucose carbon equivalents.

Resuscitation procedures

Two complementary experimental procedures were carried out (see Fig. 1). First, suspensions of nonculturable cells (VBNC and NV cells), obtained as described previously, were diluted (1 : 100) in flasks containing sterile SS, MBGYE, MBGYE supplemented with 11 U catalase mL^{-1} (MBGYEcat), DLB or DLB supplemented with catalase (DLBcat) as well as the different supernatants (final density, 10^5 cells mL^{-1}). Except sterile SS, flasks were supplemented with ciprofloxacin (0.08 μg mL^{-1}), streptomycin (64 μg mL^{-1}) and a combination of both (concentrations selected from MIC values). These antibiotics were added in order to avoid the growth of some culturable cells which might remain. Flasks were incubated with shaking at 20 or 37 °C in darkness. Daily, aliquots were collected in order to estimate TDC and CFU counts.

Moreover, resuscitation experiments were performed using the most probable number method (MPN). Decimal dilutions of nonculturable suspensions were obtained in sterile SS. One millilitre of the diluted samples was inoculated into MPN tubes containing 9 mL of MBGYE, MBGYEcat, DLB, DLBcat or supernatants and incubated at 20 or 37 °C for at least 6 days. In some cases, lysozyme

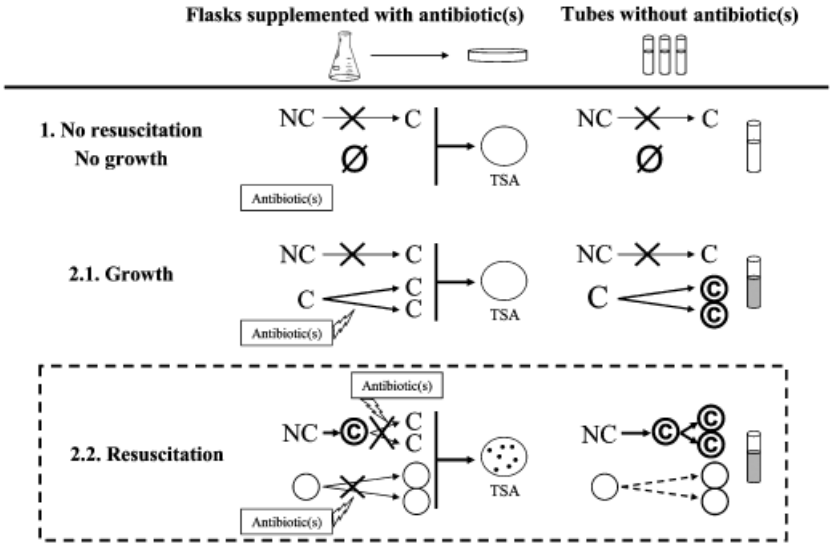

Fig. 1. Discrimination between resuscitation of nonculturable cells (NC) and growth of remaining culturable cells (C) by means of a combination of two different experimental procedures: inoculation in flasks with posterior counting in CFU plates and MPN method (see 'Material and methods'). In all cases, the nonculturable population was predominant. Ø, absence of culturable cells; C, culturable cells with a density below the detection limit of the CFU counting method used but sufficient to grow in MPN tubes; Ⓒ, culturable cells with a density sufficient to be detected both in CFU plates and MPN tubes; NC, nonculturable cells. ○, uncertainty regarding the presence of the corresponding population. Three final cases were analysed. (1) Only nonculturable cells unable to resuscitate were present. No growth in plates and tubes was observed. (2) Nonculturable cells and culturable cells (with a density below the detection limit of the CFU counting method used but sufficient to grow in MPN tubes) were present. (2.1) Nonculturable cells were unable to resuscitate. In supplemented flasks, antibiotics inhibited the culturable population from dividing and non-CFU were detected in plates. In MPN tubes in the absence of antibiotics, the culturable population divided and turbidity was observed. (2.2) NC cells were able to resuscitate. In supplemented flasks, antibiotics inhibited the culturable cells and the resuscitated nonculturable cells from dividing. However, after resuscitation, original nonculturable cells were detected in plates. In MPN tubes in the absence of antibiotics, both populations, resuscitated and culturable cells, were able to divide and turbidity was observed.

($0.1\,\mu g\,L^{-1}$) was added to the MPN tubes containing MBGYE and DLB.

MPN estimates were calculated with application of the Halvorson & Ziegler (1933) tables. Combination of results obtained from the two experimental procedures was interpreted according to Fig. 1.

Previously to resuscitation studies, flasks or tubes containing resuscitation media not supplemented with antibiotics were inoculated with decimal serial dilutions of a 100% culturable *E. coli* population or with cellular suspensions obtained from adverse conditions experiments and containing low densities of culturable cells. Incubation was done at 37 °C for at least 6 days.

Statistical analysis

Statistical analysis was carried out with the STAT VIEW program (Abacus Concept, Inc.). Values given below are the means of three experiments, and the coefficients of variation between replicates were $< 12\%$. The differences between the means were detected by a one-way ANOVA. Probabilities less than or equal to 0.05 were considered significant.

Results

Formation of nonculturable *E. coli* populations

Figures 2 and 3 show the effect of exposure to adverse conditions upon culturability, viability and integrity of *E. coli* populations as well as the percentages of nonculturable cells (VBNC and NV subpopulations). In all cases studied, the total number of bacteria (TDC) remained constant throughout the exposure while decreases in culturability (CFU) were observed.

From the decreases in CFU counts with time (Fig. 2), it was apparent that two different analyses were required: according to the beginning of the loss of culturability or to the magnitude of the phenomenon. Thus, a gradation could be achieved according to the beginning of the loss of culturability (SS at 20 °C, SW at 20 °C, SS at 37 °C, SW at 37 °C and illuminated SS at 37 °C) or to the magnitude of the loss of culturability (SW at 20 °C, SW at 37 °C, SS at 20 °C, SS at 37 °C and illuminated SS at 37 °C).

Moreover, a sharp loss of culturability was produced when 0.1% hydrogen peroxide was added to *E. coli* suspensions ($P \leq 0.05$) (Fig. 3b). Similar results were

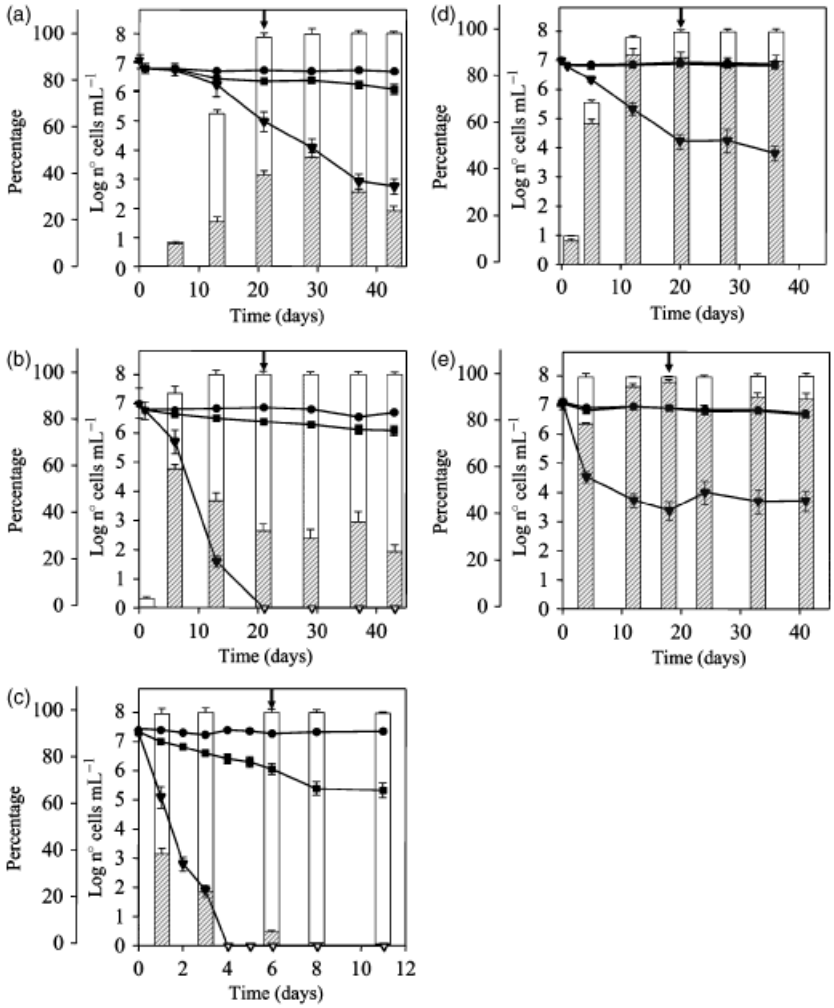

Fig. 2. Changes in bacterial counts and percentages of subpopulations during the permanence of *E. coli* cells in sterile saline solution (a–c) or seawater (d and e). Incubation at 20 °C (a, d) or 37 °C (b, c, e). (c) Exposure to visible light. Total number of bacteria (•); number of bacteria with intact cytoplasmic membranes (■), number of culturable bacteria (▼), counts below detection limit (open symbols). Percentages of viable but nonculturable (striped bars) and nonviable cells (open bars). (↓) Cells for resuscitation experiments were taken at this point. Data shown are averages of three experiments.

obtained when suspensions were acidified to pH 3 ($P \leq 0.05$) (Fig. 3a). Above this peroxide concentration or below this pH value, culturable cells were not detected.

In all cases, decreases in culturable subpopulations caused formation of NV and VBNC subpopulations. Seawater exposure produced, as a consequence of a decrease in culturability, a population mainly formed by VBNC cells (90–95% of cells). The percentage of VBNC cells did not change significantly during the course of maintenance in seawater (Fig. 2d and e) ($P > 0.05$). Under the other conditions (Figs 2a–c and 3), VBNC cells rarely formed more than 50% of the total population. Moreover, these percentages decreased during exposure to adverse conditions, increasing the NV subpopulations. At the end of maintenance in SS or for lower pH or higher concentration of peroxide tested, the NV fraction dominated the *E. coli* populations.

Resuscitation of nonculturable *E. coli* populations

Previous experimental procedures were carried out employing flasks or tubes containing resuscitation media not supplemented with antibiotics (see 'Materials and methods'). Growth of cultures was observed when at least one culturable cell was inoculated in any volume (100 or 10 mL). In those cases, TDC counts increased with incubation time and predictable instantaneous growth rates were obtained from culturable cell counts (data not shown). Thus, two antibiotics (ciprofloxacin and streptomycin, separately or in

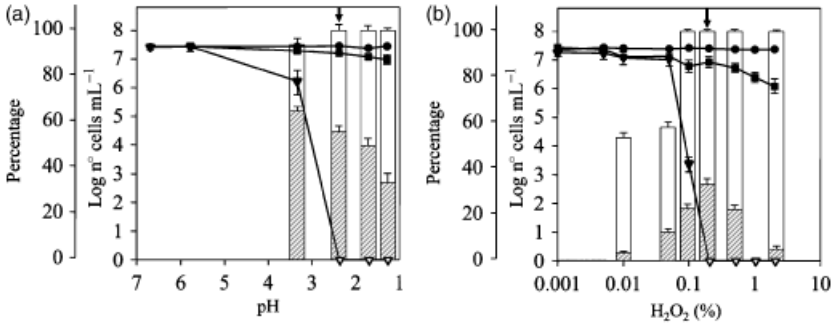

Fig. 3. Changes in bacterial counts and percentages of subpopulations during the exposure of *E. coli* cells to increasing pH (a) or hydrogen peroxide concentrations (b). Total number of bacteria (•); number of bacteria with intact cytoplasmic membranes (■), number of culturable bacteria (▼), counts below detection limit (open symbols). Percentages of viable but nonculturable (striped bars) and nonviable cells (open bars). (↓) Cells for resuscitation experiments were taken at this point. Data shown are averages of three experiments.

combination) were used to avoid growth of remaining culturable cells, with similar results.

The supernatants were, in all cases, at a pH close to neutrality (7–7.5). However, DMCH concentration showed differences depending on the culture media and on the growth phase. When the supernatants were obtained from *E. coli* or *Enterococcus faecalis* cells growing in MBGYE, the DMCH concentration was about 400 $\mu g\,mL^{-1}$ in the exponential phase and about 12 $\mu g\,mL^{-1}$ in the stationary phase. Supernatants from *P. fluorescens* in DLB had a DMCH concentration of 200 and 40 $\mu g\,mL^{-1}$ in the exponential and stationary growth phases, respectively. Moreover, nutrient concentrations in supernatants were sufficient to support *E. coli* growth after inoculation with cells from the early stationary phase of growth.

Table 1 shows results obtained when starved cells (collected from SS incubated under different conditions; 20 or 37 °C and darkness or under illumination) were submitted to resuscitation procedures at 37 °C. No changes in flasks or tubes, containing culture media, supplemented or not supplemented with catalase, or supernatants from different origin (*E. coli*, *P. fluorescens* or *Enterococcus faecalis* cultures from exponential or stationary growth phase), were detected. When the resuscitation processes were carried out at 20 °C similar results were obtained (data not shown). Thus, analysing the combined results according Fig. 1, no resuscitation and no growth were observed. Similar results were obtained from populations previously exposed to acidic conditions and to hydrogen peroxide (Table 2).

Results obtained when cells were collected after maintenance in natural seawater are shown in Table 3. Increases in cellular density were only observed when SN obtained from *E. coli* exponential cultures were used as resuscitation media. These increases (turbidity in tubes but not in antibiotic-supplemented flasks) correspond to growth of remaining culturable cells (see Fig. 1). In the other cases, neither resuscitation nor growth was observed. Similar results were obtained after resuscitation at 20 or 37 °C.

In all cases, results obtained when nonculturable populations were treated with lysozyme did not change with respect to what has been described above (data not shown) ($P > 0.05$).

Discussion

Experiments were designed to emulate the most common environmental stresses which *E. coli* could routinely encounter in diverse ecosystems. In all cases, and according to previous works (Muela *et al.*, 1999, 2000; Arana *et al.*, 2004), the population showed a pattern of response, a drop in density of culturable cells and the formation of three subpopulations (culturable, viable but nonculturable and nonviable). The relative percentages of the different subpopulations formed and the VBNC state induction period were stress-dependent (Figs 2 and 3).

In most adverse situations studied, the VBNC subpopulation did not represent the predominant fraction and decreased along the period of study. This seems to indicate that, in most situations, the VBNC is not a successful phenotype. When *E. coli* populations were exposed to adverse situations, the formation of VBNC cells would not constitute the main aim of the population.

The ability of VBNC cells to remain in and be resuscitated from this state has been reported by many authors (Whitesides & Oliver, 1997; Lleó *et al.*, 2001; Panutdaporn *et al.*, 2006). However, critical scrutiny of reported resuscitations has attributed such results to the presence of low levels of already culturable cells that simply grow (bacterial regrowth) in response to the resuscitation conditions (Ravel *et al.*, 1995; Bogosian *et al.*, 2000). A simple option to prevent the regrowth is the addition of some bacterial inhibitors to the media (Ohtomo & Saito, 2001; Wong

Table 1. Bacterial counts obtained before and after applying the resuscitation protocol based on dilution in flasks and on the MPN (tubes)

	Saline solution 20 °C				Saline solution 37 °C				Saline solution 37 °C illuminated			
	Flasks		Tubes		Flasks		Tubes		Flasks		Tubes	
Initial	TDC	CFU	TDC	MPN	TDC	CFU	TDC	MPN	TDC	CFU	TDC	MPN
	1.24×10^3	< 0.20	3.73×10^4	2.80	0.78×10^3	< 0.04	2.34×10^4	< 0.10	1.69×10^5	< 0.04	7.70×10^6	0.20
	Flasks†		Tubes		Flasks†		Tubes		Flasks†		Tubes	
Resuscitation media*	TDC	CFU	MPN		TDC	CFU	MPN		TDC	CFU	MPN	
End of resuscitation period‡												
SS	1.34×10^3	< 4.00	–		0.99×10^3	< 4.00	–		0.88×10^5	< 20.00	–	
MBGYE	1.17×10^3	< 6.25	0.70		1.10×10^3	< 6.25	< 0.30		0.69×10^5	< 20.00	0.40	
MBGYEcat	1.49×10^3	< 6.25	20.00		1.09×10^3	< 6.25	< 0.30		0.84×10^5	< 20.00	1.50	
DLB	1.30×10^3	< 6.25	2.30		1.00×10^3	< 6.25	< 0.30		0.48×10^5	< 20.00	0.40	
DLBcat	1.25×10^3	< 6.25	0.90		1.05×10^3	< 6.25	< 0.30		1.31×10^5	< 20.00	0.90	
SNexp Ec	1.95×10^3	< 4.00	2.30		0.94×10^3	< 4.00	< 0.30		–	–	–	
SNstat Ec	1.76×10^3	< 6.25	2.30		1.06×10^3	< 6.67	< 0.30		1.21×10^5	< 20.00	< 0.30	
SNexp Pf	1.61×10^3	< 6.25	23.00		0.87×10^3	< 4.00	< 0.30		0.69×10^5	< 20.00	0.40	
SNstat Pf	1.87×10^3	< 6.25	0.90		1.87×10^3	< 6.25	< 0.30		–	< 20.00	0.30	
SNexp Ef	1.45×10^3	< 6.25	4.00		1.01×10^3	< 6.25	< 0.30		1.79×10^5	< 20.00	0.40	
SNstat Ef	1.80×10^3	< 4.00	4.00		1.80×10^3	< 6.25	< 0.30		0.82×10^5	< 20.00	< 0.3	

*SS, saline solution; MBGYE, M9 broth supplemented with 0.05% glucose and with 0.03% yeast extract; DLB, diluted Luria–Bertani broth; SNexp and SNstat, supernantants from exponential and stationary phase of growth. cat, catalase supplementation; Ec, Pf and Ef, the microorganism from which the supernatant was derived, *E. coli*, *Pseudomonas fluorescens* or *Enterococcus faecalis*, respectively.

†Flasks supplemented with ciprofloxacin. Flasks filled with saline solution (SS) were not supplemented.

‡Resuscitation carried out at 37 °C.

Nonculturable populations were collected after starvation of cells in saline solution incubated at 20 or 37 °C in darkness or under illumination.

et al., 2004). Moreover, in this study, when at least one culturable cell was inoculated in any volume (100 or 10 mL) of nonantibiotic-supplemented resuscitation media, population increases were observed. From the calculated instantaneous growth rate, the increases in population observed were justified from growth of culturable cells. Oliver (2005) and Wai *et al.* (2000) recommend the dilution method in order to preclude the possibility of any culturable cells being present and growing. By combining these methods we have designed a working protocol (see Fig. 1) in order to distinguish unequivocally between regrowth and resuscitation.

The first resuscitation factor considered by several authors, regardless of the microorganism studied, is the removal of environmental stress (Magariños *et al.*, 1997; Whitesides & Oliver, 1997; Ohtomo & Saito, 2001; Gupte *et al.*, 2003). In this study, we have obtained no indication that the nonculturable cells could be resuscitated by the reversion of those abiotic factors which induced nonculturability (see Tables 1–3).

The elevated concentrations of nutrients in the resuscitation media might be toxic to cells in the VBNC state (Dukan *et al.*, 1997; Whitesides & Oliver, 1997), producing superoxide and free radicals (Wong *et al.*, 2004). To prevent this additional oxidative stress, supplementation with oxidant-degrading agents has been recommended (Wai *et al.*, 2000). However, Bogosian *et al.* (2000) and Wong *et al.* (2004) have indicated that increases in culturable cells are probably due to recuperation of injured cells instead of resuscitation of VBNC cells. Our results have shown, for the first time, that the addition of those compounds to the enumeration or resuscitation media did not provoke the resuscitation of VBNC cells.

The second category of treatments to induce resuscitation would include the activation of replicative function by stimulation of VBNC cells (Wai *et al.*, 1996; Gupte *et al.*, 2003). Peptidoglycan hydrolysis has been also suggested to be required for the activation of VBNC cells. This would facilitate cell division (Signoretto *et al.*, 2002) or the release of lysis products acting as resuscitation signals (Keep *et al.*, 2006b). In order to prove this, VBNC *E. coli* cells were exposed to lysozyme without any effect on recuperation of culturability.

Supernatants from different growth phases and microorganisms, such as those tested in our resuscitation experiments, contain nutrients and, possibly, signal molecules. Nutrient concentrations, estimated approximately as DMCH content, were at least 14-fold higher than those

Table 2. Bacterial counts obtained before and after applying the resuscitation protocol based on dilution in flasks and on the MPN (tubes)

	pH 2.6				0.2% Hydrogen peroxide			
	Flasks		Tubes		Flasks		Tubes	
Initial	TDC	CFU	TDC	MPN	TDC	CFU	TDC	MPN
	2.28×10^5	< 4.00	2.19×10^6	3	2.43×10^5	< 4.00	2.47×10^6	< 0.30
	Flasks†		Tubes		Flasks†		Tubes	
Resuscitation media*	TDC	CFU	MPN		TDC	CFU	MPN	
End of resuscitation period‡								
SS	2.06×10^5	< 4.00	–		1.75×10^5	< 4.00	< 0.30	
MBGYE	1.17×10^5	< 4.00	4.00		1.93×10^5	< 4.00	< 0.30	
MBGYEcat	1.49×10^5	< 4.00	2.10		1.39×10^5	< 4.00	< 0.30	
DLB	1.33×10^5	< 4.00	2.30		2.19×10^5	< 4.00	< 0.30	
DLBcat	1.85×10^5	< 4.00	0.90		2.51×10^5	< 4.00	< 0.30	
SNexp Ec	1.76×10^5	< 4.00	–		1.92×10^5	< 4.00	< 0.30	
SNstat Ec	2.12×10^5	< 4.00	< 0.30		–	–	–	
SNexp Pf	1.61×10^5	< 4.00	21.00		1.86×10^5	< 4.00	< 0.30	
SNstat Pf	0.88×10^5	< 4.00	–		2.20×10^5	< 4.00	< 0.30	
SNexp Ef	1.55×10^5	< 4.00	–		1.59×10^5	< 4.00	< 0.30	
SNstat Ef	2.06×10^5	< 4.00	–		2.34×10^5	< 4.00	< 0.30	

*Resuscitation media: SS, saline solution; MBGYE, M9 broth supplemented with 0.05% glucose and with 0.03% yeast extract; DLB, diluted Luria–Bertani broth; SNexp and SNstat, supernantants from exponential and stationary phase of growth. cat, catalase supplementation; Ec, Pf and Ef, the microorganism from which the supernatant was derived, *E. coli*, *Pseudomonas fluorescens* or *Enterococcus faecalis*, respectively.

†Flasks supplemented with ciprofloxacin. Flasks filled with saline solution (SS) were not supplemented.

‡Resuscitation carried out at 37 °C.

Nonculturable populations were collected after exposure of cells to pH 2.6 or 0.2% hydrogen peroxide.

which support growth in supernatants from survival experiments (Arana *et al.*, 2004). In all cases, regardless of the origin of supernatant, the resuscitation was unsuccessful, indicating the absence of signal molecules conducting the resuscitation process, as well as the inability of the environmental stress removal to resume culturability.

The resuscitation process has been extensively studied in several bacteria (Mukamolova & Kormer, 1999; Lleó *et al.*, 2000, 2001; Gupte *et al.*, 2003; Smith & Oliver, 2006) and factors promoting resuscitation are well established. Studies regarding *E. coli* resuscitation are scattered and offer contradictory results (Bogosian *et al.*, 1996, 1998; Dukan *et al.*, 1997; Ohtomo & Saito, 2001). In this work, we have studied, for the first time, the resuscitation process and the factors promoting it (Yamamoto, 2000) as a whole.

Repeated resuscitation attempts over the course of this study indicate that the *E. coli* strain used is not able to resuscitate from the VBNC state. Moreover, from the survival experiments, we can conclude that VBNC phenotype does not appear to be successful because the VBNC subpopulation does not represent the predominant fraction and it decreases along the period of study.

The behaviour of *E. coli* should not necessarily be considered a standard for other bacteria. Alternation of favourable and unfavourable situations is typical in aquatic ecosystems. For autochthonous bacterial species as *Vibrio vulnificus*, entry into the VBNC state and subsequent resuscitation in response to changing environmental conditions could be a strategy which contributes to ensure their perpetuation (Oliver, 2005). However, we should take into consideration that *E. coli* lives, in high-density populations, in the relatively stable environment of the intestine. Therefore, it is probable that its genetic endowment does not include a programme in which resuscitation constitutes a strategy for the perpetuation of the species.

We have previously stated that during survival under adverse conditions, organic substances (proteins, dissolved free amino acids, DMCH) are released into the surrounding medium (Arana *et al.*, 2004). We hypothesized that the turnover of these molecules in the medium could be sufficient to repair lesions and to retard cell deterioration. A similar suggestion has been proposed by Cuny *et al.* (2005). Aertsen & Michiels (2004) and Lewis (2000) propose that in the case of serious damage by stressing factors, cells will donate their nutrients to other members of the population to ensure species survival until better environmental conditions. The surviving cells can cheat at the expense of the altruism displayed by the majority. Thus, the nutrients left over or contributed by the VBNC cells could serve to aid the survival of the persisting culturable cells. We can

Table 3. Bacterial counts obtained before and after applying the resuscitation protocol based on dilution in flasks and on the MPN (tubes)

	Seawater 20 °C				Seawater 37 °C			
	Flasks		Tubes		Flasks		Tubes	
Initial	TDC	CFU	TDC	MPN	TDC	CFU	TDC	MPN
	1.24×10^3	< 20.00	1.68×10^4	< 10.00	7.98×10^3	21.00	1.60×10^3	4.20
	Flasks[†]		Tubes		Flasks[†]		Tubes	
Resuscitation media*	TDC	CFU	MPN		TDC	CFU	MPN	
End of resuscitation period[‡]								
SS	1.68×10^4	< 10.00	–		5.22×10^3	6.00	–	
MBGYE	4.67×10^4	< 10.00	7.00		2.78×10^3	< 1.00	11.00	
MBGYEcat	–	–	20.00		2.34×10^3	< 1.00	11.00	
DLB	1.00×10^4	< 10.00	4.00		2.34×10^3	< 1.00	5.00	
DLBcat	3.95×10^4	< 10.00	4.00		1.39×10^3	< 2.00	2.10	
SNexp Ec	1.40×10^4	< 10.00	4.00		1.79×10^3	< 2.00	G[§]	
SNstat Ec	1.99×10^4	< 10.00	G[§]		2.14×10^3	13.00	11.00	
SNexp Pf	1.04×10^4	< 10.00	4.00		2.14×10^3	< 2.00	5.00	
SNstat Pf	0.85×10^4	< 10.00	1.10		3.48×10^3	14.00	0.90	
SNexp Ef	1.01×10^4	< 10.00	4.00		2.88×10^3	< 2.00	5.00	
SNstat Ef	–	–	–		–	–	G[§]	

*Resuscitation media: SS, saline solution; MBGYE, M9 broth supplemented with 0.05% glucose and with 0.03% yeast extract; DLB, diluted Luria–Bertani broth; SNexp and SNstat, supernantants from exponential and stationary phase of growth. cat, catalase supplementation; Ec, Pf and Ef, the microorganism from which the supernatant was derived, *E. coli*, *Pseudomonas fluorescens* or *Enterococcus faecalis*, respectively.

[†]Flasks supplemented with ciprofloxacin. Flasks filled with saline solution (SS) were not supplemented.

[‡]Resuscitation carried out at 37 °C.

[§]Growth.

Nonculturable populations were collected after permanence of cells in seawater incubated at 20 or 37 °C in darkness.

conclude that the formation of a VBNC subpopulation could be seen as an adaptive process, designed for the benefit of the population as a whole.

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