Effect of temperature, salinity and nutrient content on the survival responses of Vibrio splendidus biotype I

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The aim of this study was to evaluate the survival responses of two strains of Vibrio splendidus, both in natural and in defined media. For this purpose, freshwater and defined media containing different salinities (3–3-0.9%) and nutrient concentrations (17–0.005 mg l⁻¹) were assayed. The incubation temperatures were established at 4, 10 and 22 °C. The acridine orange staining technique was used for total cell enumeration and the number of viable cells was determined using two direct assays, nalidixic acid and tetrazolium salt reduction and plate spreading. Resuscitation assays of viable but non-culturable (VBNC) cells were conducted. According to the counting procedures employed, at least four different subpopulations were found: (i) active (positive response in both nalidixic acid and tetrazolium assays) culturable cells; (ii) active non-culturable cells; (iii) tetrazolium-salt-responsive non-culturable cells and (iv) non-active (responsive to none of the direct viable assays) non-culturable cells. Long-term survival was found at salinities and nutrient concentrations of seawater environments (3–3% and 5 mg l⁻¹ or 1 g l⁻¹), whereas the strains entered a VBNC state in freshwater and in brackish (0.9 or 1.6% salinities) or high nutrient content (17 g l⁻¹) defined medium. The recovery of VBNC cells was not achieved.

INTRODUCTION

Since the first description of Vibrio splendidus (Reichalt et al., 1976), this species has been reported as the causal agent of disease in fish and shellfish (Angulo et al., 1994a, b; Miranda & Rojas, 1996; Balebona et al., 1998). Several studies have been conducted on its isolation from natural habitats (West et al., 1986; Ortigosa et al., 1994; López & Angulo, 1995; Farto et al., 1999). Environmental studies have revealed the existence of significant differences between total counts, direct viable counts and plate counts in samples taken from natural aquatic environments (Bianchi & Giuliano, 1996). To overcome this Roszik & Colwell (1987) defined a physiological state in which microorganisms are unable to grow on solid media, but show other functions characteristic of living cells, such as substrate responsiveness (Kogure et al., 1979), respiration (Zimmermann et al., 1978), membrane potential (Smith, 1990) and enzymic activity (Morr et al., 1988; Nwobugh et al., 1995). This state was termed ‘viable but non-culturable’ (VBNC), with at least ten Vibrio species being described as having the potential for it [Vibrio (now Listonella) anguillarum, Vibrio campbellii, Vibrio cholerae, Vibrio fischeri, Vibrio harveyi, Vibrio mimicus, Vibrio natriegens, Vibrio paraheamolyticus, Vibrio proteolyticus, Vibrio vulnificus; McDougald et al., 1998]. Different authors have proposed that the VBNC state is an adaptive strategy of micro-organisms against stress from which cells should be able to recover once optimal conditions are restored (Huq & Colwell, 1995; Nybroe, 1995; Oliver, 1995). However, although for some organisms resuscitation has been found to be possible under certain conditions (V. cholerae 01, Colwell et al., 1996; V. vulnificus, Oliver & Bockian, 1995; enteropathogenic Escherichia coli, Pommpeuy et al., 1996; Shigella dysenteriae, Rahman et al., 1994) it has been found to be impossible for some others (V. paraheamolyticus; Jiang & Chai, 1996).

The role of the VBNC state on the ecology and life-cycle of different bacterial species is being evaluated (Lebaron et al., 1999; Grimes et al., 2000; Huq et al., 2000). Current research is strongly guided by evidence of the existence of different cellular physiological states that marine bacteria display under variable environmental conditions (Kaprelyants et al., 1996; Comas & Vives-Rego, 1998; Fegatella & Cavicchioli, 2000).

The aim of the present work was to study the effect of temperature, salinity and nutrient concentration on the survival responses of V. splendidus, using both natural freshwater and defined medium laboratory microcosms.
Also resuscitation experiments were conducted, to establish the growth-limiting natural conditions of this species.

**METHODS**

**Bacterial strains and culture conditions.** *V. splendidus* strains 16N and 43N were isolated as the causal agents of different vibriosis outbreaks affecting cultured turbot (Angulo et al., 1994a, b) and were taxonomically confirmed by Montes et al. (1999). The original strains were maintained at −80 °C in tryptone soy broth (Adams-Micro) supplemented with 1.5% (w/v) NaCl (Panreac) and 15% (v/v) glycerol (Panreac), and routinely cultivated on tryptone soy agar (Prondisa), supplemented with 2% NaCl at 22 °C for 48 h. Cultures of exponentially growing cells were inoculated into 150 ml microcosms containing either freshwater or defined medium at final densities of 10^5 cells ml⁻¹. All the experiments were performed in triplicate.

The freshwater was collected from a public fountain in Vigo (chemically untreated; pH 6-16), sterilized through 0.22 μm filters and placed into asptic flasks.

The defined media were based on those described by Weichart & Kjelleberg (1996), although three different nutrient and salt concentrations were prepared. Nutrients were aseptically added to the microcosms at final concentrations of 17 or 1 g l⁻¹, or 5 mg l⁻¹, each one containing 49-8% peptone (Cultimed), 24-9% yeast extract (Cultimed), 24-9% glucose (Panreac) and 0-4% Na₂HPO₄ (Panreac). Iron (FeSO₄.7H₂O; Panreac), prepared in 0-4 M Tricine (Sigma), was added from a filter-sterilized solution at a final concentration of 50 mM. A 3.3% salinity stock solution was prepared with (g l⁻¹) NaCl (29-4), Na₂SO₄ (2-4), NaHCO₃ (0-1), KCl (0-4), KBr (0-07), MgCl₂·6H₂O (3-1), CaCl₂·2H₂O (0-7), SrCl₂ (0-013) and H₂BO₃ (0-013) (all purchased from Panreac). Two additional 1-6 and 0-9% salinity working solutions were obtained by 5 × 10⁻¹ and 2.5 × 10⁻¹ dilutions of the initial stock. The salinities were measured with a portable salinometer (Orion 130). The survival experiments were performed at 4, 10 or 22 °C.

**Nalidixic acid direct counts (NADC) and acridine orange total counts (AOTC).** Duplicate samples from the microcosms were mixed with both nalidixic acid (NA; Sigma) and yeast extract (Oxoid) at final concentrations of 0-02% (w/v) and incubated overnight at 22 °C in darkness with shaking at 100 r.p.m. (modified from Kogure et al., 1979). NA-treated and untreated (control) samples were then stained with acridine orange (Sigma) at a 0.01% (w/v) final concentration for 2 min. Samples were filtered through 0.22 μm pore-size black polycarbonate filters (Millipore). Counts were performed in an Olympus BH2-RFC fluorescence microscope (blue-light excitation; 1250 × final magnification) with a micro-meter eyepiece. The length of 250–400 control cells was measured and the mean value was calculated. NA-active cells were recorded as those which were elongated at least 1.5-fold in length with respect to the control mean length (Barcina et al., 1995). AOTC and NADC were counted in 40 fields using a 10 × 10 ocular grid and the mean number of cells per field was obtained.

**Tetrazolium salt direct counts (TSDC).** Aliquots from the microcosms were incubated with 0-01% (w/v) of the tetrazolium salt (TS) p-iodonitrophenyltetrazolium chloride (Sigma) at 22 °C for 1 h, in darkness and with shaking at 100 r.p.m. (Zimmermann et al., 1978). The samples were fixed with 2% (w/v) formaldehyde and placed in a counting chamber (Neubauer Improved). Counts were performed in duplicate using an Olympus BH2 microscope (1250 × final magnification). Those cells containing an intracellular reddish-brown formazan spot were recorded as TS-responsive.

**Plate counts (PC).** The number of c.f.u. was determined by duplicate plating on tryptone soy agar (TSA) and 5 × 10⁻² and 2.5 × 10⁻⁴ dilutions of this medium. These media were amended with NaCl to mimic the salinity of the microcosms. After 2–3 days at 22 °C, the plates were inspected for c.f.u. determination. The experiments in which culturable cells could still be detected after a minimum of 3 months were stopped.

**In vitro resuscitation assays.** The resuscitation experiments were started once the culturable cell numbers were fixed at a level below 0-1 cells ml⁻¹. To attain this value, once PC reached zero by 0.1 ml seeding, cells from 10 ml samples were collected onto sterile 0.2 μm cellulose nitrate filters (Albet). These filters were then placed on TSA, 5 × 10⁻² TSA and 2.5 × 10⁻⁴ TSA (with the appropriate NaCl concentration) and plates were incubated for 1 week at 22 °C. Afterwards, samples of 1 and 2 ml were added in duplicate to tubes containing 9 or 2 ml (double-strength), respectively, fresh liquid medium. TSA and 5 × 10⁻² and 2.5 × 10⁻⁴ dilutions of this medium with the appropriate NaCl concentration were used. After 1 week at 22 °C with shaking, the presence of turbidity was recorded as a positive result.

**Statistical analysis.** The statistical analysis was performed using the SPSS 10.0 program. The survival times of the strains and the counting procedures were compared two by two using one-way variance analysis (ANOVA), including temperature, salinity and nutrient content as co-variables. The influence of these variables on the survival time of the strains was evaluated by regression analysis. Probability values lower than or equal to 0.05 were considered statistically significant. The mean values of duplicate samples and triplicate experiments for all the counting procedures were used for graphic representation.

**RESULTS**

**Influence of temperature, salinity and nutrient concentration on the survival responses of *V. splendidus* biotype I**

A total of three natural freshwater and seven defined media microcosms were assayed for each strain. The survival times of the strains of *V. splendidus* biotype I (determined as the mean survival time in the three solid media employed) ranged from zero to more than 130 days (Table 1). When the survival times of both strains were compared by one-way ANOVA, no differences were found between data. However, it was found that strains 16N and 43N greatly differed in the time they remained culturable in two of the defined medium microcosms. Strain 16N lost culturability after 21 days at 3.3% salinity, 1 g l⁻¹ of nutrient content and incubation temperatures of 4 or 10 °C, while in these same microcosms, strain 43N persisted in a culturable state for more than 124 days (Table 1).

The mean survival time of the strains of *V. splendidus* was found to be statistically related to the salinity and nutrient concentration in the defined medium microcosms according to the equation

\[
t_{\text{surv}} = \frac{k \times 29.3 \times S - 3.6 \times N}{2.5 \times 10^{-4}}
\]

where \(t_{\text{surv}}\) is the mean survival time (days); \(k\) is a constant, \(S\) is salinity (%), \(N\) is nutrient concentration (g l⁻¹) and \(R^2\) is the correlation coefficient. This qualitative relation was...
ascertained as positive with salinity, whereas it proved to be negative with nutrient content. The hypothesis thus proposed would predict a higher persistence of the strains of *V. splendidus* with higher salinities and lower nutrient concentrations present in the defined media. The temperatures assayed were not found to affect the survival of the strains.

Strains 16N and 43N maintained under controlled laboratory conditions led to the development of at least four subpopulations, which differed in their culturability and activity. These subpopulations were composed of (i) NA- and TS-responsive (active) culturable cells, (ii) active non-culturable cells; (iii) only TS-active non-culturable cells and (iv) non-active non-culturable cells. Subpopulations (ii) and (iii) are referred to as VBNC.

When strains 16N and 43N were maintained in the defined medium of 3-3% salinity and nutrient concentrations of 1 g l\(^{-1}\) or 5 mg l\(^{-1}\), the populations remained homogeneous and only subpopulation (i) could be detected (Fig. 1). During the time in which population assessment was carried out, AOTC remained constant, except for an initial increase due to nutrient availability, and cell populations remained both active and culturable. After a minimum of 96 and a maximum of 130 days of maintained culturability the experiments were terminated (Table 1). In some of these long-term culturable microcosms, NADC dropped below the limits of detection before both PC and TSDC (Fig. 1a). The registered survival times ranged from 0 to 61 days (Table 1) and three different subpopulations were observed once populations became non-culturable: cells showing both the NA-response and TS-reducing ability [subpopulation (ii)], TS-reducing cells [subpopulation (iii)]

### Table 1. Survival time of the strains 16N and 43N of *V. splendidus* maintained in freshwater and defined medium microcosms

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>Salinity (%)</th>
<th>Nutrient concentration (g l(^{-1}))</th>
<th>Survival time (days)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>96</td>
</tr>
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*ND, Not determined.
†Mean value of the survival times obtained in the three solid media and in triplicate experiments.
‡Time at which PC were stopped in long-term culturable microcosms.

Strains 16N and 43N lost the ability to form colonies on solid media in freshwater microcosms and in the defined medium microcosms of 1-6 and 0.9% salinity. Cells only entered a VBNC state in the defined medium microcosm of 3-3% salinity containing the highest nutrient concentration (17 g l\(^{-1}\)). The registered survival times ranged from 0 to 61 days (Table 1) and three different subpopulations were observed once populations became non-culturable: cells showing both the NA-response and TS-reducing ability [subpopulation (ii)], TS-reducing cells [subpopulation (iii)]

![Fig. 1](http://mic.sgmjournals.org)
or cells lacking a positive response in both assays [subpopulation (iv)]. Thus 16N and 43N cell populations kept in the 0-9 % salinity defined medium with 1 g l⁻¹ of nutrient content at 10 °C retained activity in NA and TS assays, even 14 days after the ability to grow on solid medium was lost [subpopulation (ii); Fig. 2]. However, when the defined medium of 1-6 % salinity was assayed [subpopulation (iii)] NADC dropped below detectable values at the same time as or after PC did (Fig. 3a, b). In spite of this, the respiratory activity was maintained. Strain 16N cultured in freshwater at 10 °C resulted in undetectable NA- and TS-active cells after 0 and 7 days, respectively [subpopulation (iv)], in Fig. 4.

Finally, when the behaviour of strain 43N was studied in freshwater at 22 °C the decay of both NA and TS activities was followed by a decrease of two logarithmic units in total cell numbers (Fig. 5). This would clearly seem to point to the fact that part of the population has undergone cellular lysis.

**Analysis of the counting procedures**

When the different counting procedures were compared and analysed, PC obtained in TSA, 5×10⁻² TSA and 2.5×10⁻⁴ TSA were found to be statistically identical. These results confirm the usefulness of the three solid media employed for the determination of the culturable cell numbers, even when a 2.5×10⁻⁴ dilution of the original medium was used. Furthermore, it was found that NADC were statistically equal to the mean value of PC (mean value of PC obtained on the three solid media) and lower than TSDC (P=0.00).

**Fig. 2.** Survival of strains 16N (a) and 43N (b) of *V. splendidus* at 10 °C in the defined medium of 0-9 % salinity and 1 g l⁻¹ nutrient concentration. Filled circle, AOTC; filled square, NADC; filled triangle, TSDC; open triangle, PC obtained in TSA; open square, PC obtained in 5×10⁻² TSA; open triangle, PC obtained in 2.5×10⁻⁴ TSA. Each point represents the mean of two determinations and three independent experiments (SD <12 %).

**Fig. 3.** Survival of strains 16N (a) and 43N (b) of *V. splendidus* at 10 °C in the defined medium of 1-6 % salinity and 1 g l⁻¹ nutrient concentration. Filled circle, AOTC; filled square, NADC; filled triangle, TSDC; open triangle, PC obtained in TSA; open circle, PC obtained in 5×10⁻² TSA; open square, PC obtained in 2.5×10⁻⁴ TSA. Each point represents the mean of two determinations and three independent experiments (SD <12 %).

**Fig. 4.** Survival of strain 16N at 10 °C in freshwater. Filled circle, AOTC; filled square, NADC; filled triangle, TSDC; open triangle, PC obtained in TSA; open circle, PC obtained in 5×10⁻² TSA; open square, PC obtained in 2.5×10⁻⁴ TSA. Each point represents the mean of two determinations and three independent experiments (SD <12 %).
Vibrio splendidus, years. On the other hand, ability in seawater, with survival times ranging from 1 to 3 days. Other species like V. splendidus to those obtained for the strains ATCC 33125 and 43N of V. vulnificus biotype I displayed several survival strategies showing the efficiency of the species to maintain itself as culturable at low temperatures.

Longest survival times were recorded when these para-culturable state in the defined medium microcosms. The temperatures did not affect the survival of Salmonella typhimurium in laboratory microcosms when challenged with different environmental conditions. Joux and others have influenced the ability of V. splendidus to persist in a VBNC state in freshwater (Lo´ pez & Angulo, 1995). The studied V. (now Listonella) anguillarum, Vibrio salmonicida and V. vulnificus demonstrated a long survival ability in seawater, with survival times ranging from 1 to 3 years. On the other hand V. splendidus maintained in brackish defined medium microcosms (salinities of 0.9 and 1.6 %), freshwater or with 17 g l\(^{-1}\) of nutrient content entered a VBNC state. Non-culturability of Vibrio species are formed over a wide range of salinities (Hoff, 1989; Kim & Kwon, 1997; Marco-Noales et al., 1999).

Also, high nutrient content has been found to induce a VBNC state in V. vulnificus (Oliver, 1995). The studied temperatures did not affect the survival of V. splendidus, showing the efficiency of the species to maintain itself as culturable at low temperatures.

V. splendidus biotype I displayed several survival strategies in laboratory microcosms when challenged with different environmental conditions. Joux et al. (1997) described the death kinetics of starved Salmonella typhimurium as a process beginning with cells losing both the ability to grow on solid medium and to respond to the NA assay, at the same time. Later cells lost the respiratory activity measured by a TS reduction and finally, cellular integrity. Although the compounds used to measure the respiratory activity were not the same, this sequence in the decay of cell activities was only observed when strain 43N was cultured in freshwater at 22 °C. In this study we have found that stressful conditions produce different responses in V. splendidus.

According to Villarino et al. (2000) cells in the VBNC state could be metabolically reactivated. In our case, resuscitation of VBNC populations was not achieved. Although only variations in the temperature and nutrient concentrations were assayed, some authors have pointed out that reversal of the inducing factor is not always enough to produce a return to culturability (Ghezzi & Steck, 1999).

With respect to direct viable counting procedures, TSDC and NADC have been widely used for assessing cell activities in several environmental studies (Huq et al., 2000) and different authors have found a good correlation between values obtained by both procedures (Wolf & Oliver, 1992). In our study, the statistical comparison of TSDC and NADC has revealed higher yields of the former, indicating that these assays measure different cellular activities. As pointed out by Joux et al. (1997), NADC and PC measure a potential growth ability, as a result of nutrient addition. NA blocks the activity of the DNA gyrase, thus inhibiting cell division. However, TSDC measure a real respiratory activity.

In conclusion, although the results stated above are not directly extrapolable to natural habitats, salinities of marine environments produce long-term survival responses, whereas those of freshwater or estuarine areas and/or high organic matter concentrations lead to the formation of a VBNC state in V. splendidus. In all but one of the experiments heterogeneity within populations was found, instead of a programmed sequence leading to cell death. However, the recovery of non-culturability populations was not attained by in vitro procedures. These results suggest that the VBNC state is an adaptation strategy in V. splendidus and the isolation of this species from its natural habitat can be unsuccessful.

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REFERENCES


