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Long-term survival and the viable but nonculturable state as part of the life cycle of *Listonella pelagia*

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ABSTRACT: Survival of the turbot-virulent strain 7P of *Listonella pelagia* was evaluated at 3 incubation temperatures (4, 10 and 22°C) in microcosms of natural freshwater and defined media of different salinities (9, 16 and 33‰) and nutrient concentrations (0.005, 1 and 17 g l^{-1}). During the experimental period, different counting procedures were used to assess the population state. These were: (1) acridine orange total counts (AOTC); (2) nalidixic acid direct counts (NADC); (3) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) direct counts (INTDC); and (4) plate counts (PC). Resuscitation of the viable but nonculturable (VBNC) populations was performed both *in vitro* and *in vivo*. Electron microscopy and membrane protein profiling of the cells was conducted. Irrespective of the temperatures assayed, *L. pelagia* remained culturable for long periods in the defined-medium microcosms of 33‰ and 0.005 or 1 g l^{-1} nutrient content, but entered a VBNC state in freshwater, at lower salinities (9 or 16‰) and/or at the highest nutrient content (17 g l^{-1}). Although, in these cases, cells displayed a spheroid morphology, a significant reduction in size ($p < 0.05$) was only observed in long-term surviving cells. No changes were found in membrane protein profiles. VBNC cells were recovered *in vitro* in samples that contained less than 0.0001 culturable cells ml^{-1}, or that had been treated with ampicilline at bacteriolytic concentrations, and from the internal organs of infected sea bream. The overall results suggest long-term survival of *L. pelagia*, and that the VBNC state is part of its life cycle.

KEY WORDS: *Listonella pelagia* · Long-term survival · Viable but nonculturable state · *In vitro* · *In vivo* · Resuscitation

INTRODUCTION

Listonella pelagia (MacDonell & Colwell 1985), formerly classified as *Vibrio pelagius* (Baumann & Baumann 1980), is a marine halophilic bacteria reported to be an opportunistic pathogen affecting various fish and shellfish species (Angulo et al. 1992, Castro et al. 1992, Santos et al. 1997).

Different studies have shown that under certain conditions, marine microorganisms can enter 2 different physiological states (Oliver 1993). Oligotrophic environments, such as oceanic areas, induce the starvation survival response, where the formation of ultramicrocells and the synthesis of 'cross-protective' stress proteins have been demonstrated (Kjelleberg et al. 1993, Fegatella & Cavicchioli 2000). In the 'viable but nonculturable' (VBNC) state, microorganisms — although they display other measurable life activities — cannot be detected by growth on bacteriological media (Roszak & Colwell 1987). Whether the VBNC state is a survival strategy against stressful environmental conditions (Oliver et al. 1995, Huq et al. 2000), or a process of deterioration leading to cell death (Desnues et al. 2003), is yet to be determined. The first hypothesis implies reversibility and has to preclude the possibility of regrowth by a few remaining culturable cells. High dilution rates, or inhibitors of these remaining actively growing cells, have been used to address this hypothesis (Lleo et al. 1998). However, (1) controversial resuscitation results have been found even within the same species (Wai et al. 2000, Rahman et al. 2001) and (2) reversal of the inducing factor is not always sufficient to induce a return to culturability and, therefore, recovery conditions may be difficult to

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find. In fact, several reports indicate that the return to culturability is stimulated by autocrine or extracellular factors (Steinert et al. 1997, Biketov et al. 2000). Furthermore, for the VBNC state to be considered as a programmed response, it is necessary to find the genes involved (Chowdbury et al. 1994). Also, oxidative stress has been associated with the VBNC state. After adding hydrogen-peroxide-degrading compounds to resuscitation media, Bogosian et al. (2000) found the apparent recovery of *Vibrio vulnificus*.

Since the first description of the VBNC state, a reduction in cell size and a morphological change from rod to coccus has been accepted for most (Oliver 2000), if not all, species (Mizunoe et al. 2000, Mary et al. 2002). Moreover, alterations in the cell wall, membrane, cytoplasm, protein, RNA and DNA synthesis have been described (Huq et al. 2000, Oliver 2000, Chaiyanan et al. 2001, Signoretto et al. 2002). The potential virulence of VBNC cells has been demonstrated for various human (Rahman et al. 1994, Pommepuy et al. 1996, Sack et al. 1998), plant (Grey & Steck 2001) and fish pathogens (Magariños et al. 1994), although in some other cases this could not be verified (Forsman et al. 2000, Hald et al. 2001).

The aim of this work was to assess the influence of different environmental variables on the survival of a turbot virulent strain of *Listonella pelagia* in order to establish the ability of this opportunistic pathogen to disseminate itself in freshwater and estuarine environments, and to evaluate the possible epizootic risks. Cell populations were characterized by acridine orange total counts (AOTC), nalidixic acid direct counts (NADC), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) direct counts (INTDC) and plate counts (PC). Cells were examined by electron microscopy and their membrane protein profiles studied. Furthermore, the significance of the VBNC in this species was evaluated both by *in vitro* and *in vivo* resuscitation assays.

MATERIALS AND METHODS

Strain and culture conditions. Strain 7P of *Listonella pelagia* was isolated from the internal organs of diseased juvenile turbot reared on a fish farm on the Ría de Vigo (NW Spain). The strain showed an LD_{50} of 9.5×10^4 colony-forming units (cfu) after intraperitoneal injection into turbot weighing 5 g (Angulo et al. 1992). The original strain was preserved by freezing at –80°C in tryptone soy broth (TSB), with a final concentration of 2% sodium chloride (NaCl) and 15% glycerol. Routine cultures were obtained on tryptone soy agar (TSA) supplemented with 1.5% NaCl (TSA-2%) for 48 h at 22°C. Exponentially growing cells were transferred to flasks containing 200 ml of fresh water or defined medium at approximate final densities of 10^6 cells ml^{-1}. Fresh water was collected from a public fountain in Vigo (chemically untreated; pH 6.16), sterilized through 0.22 µm filters and placed into aseptic flasks.

Defined media were composed of nutrient, salts and iron solutions. Three different nutrient and salt concentrations were prepared (modified from Weichart & Kjelleberg 1996). The nutrient solution was aseptically added to the microcosms to obtain final concentrations of 0.005, 1 or 17 g l^{-1}, each containing 49.75% peptone, 24.88% yeast extract, 24.88% glucose and 0.5% Na_2HPO_4. The 33‰ microcosms contained 29.4 g NaCl l^{-1}, 2.4 g Na_2SO_4 l^{-1}, 0.1 g $NaHCO_3$ l^{-1}, 0.4 g KCl l^{-1}, 0.07 g KBr l^{-1}, 3.1 g $MgCl_2 \cdot 6H_2O$ l^{-1}, 0.7 g $CaCl_2 \cdot 2H_2O$ l^{-1}, 0.013 g $SrCl_2$ l^{-1} and 0.013 g H_3BO_3 l^{-1}. The 16 and 9‰ microcosms were prepared with ½ and ¼ of these quantities, respectively. The salinities were measured with a portable salinometer (Orion 130). Finally, a filter-sterilized iron solution ($FeSO_4 \cdot 7H_2O$ in 0.4 M tricine) was added at a final concentration of 0.01 mM. Survival experiments were performed in duplicate at incubation temperatures of 4, 10 or 22°C while rotating (100 rpm).

Cell enumeration procedures. At the time of inoculation and at regular intervals after that, samples were removed from microcosms in order to perform the different counting procedures. After colonies could no longer be detected on solid media, direct viable counts (NADC, INTDC) were extended to continue over a period of 1 or more weeks.

AOTC and NADC: Duplicate 0.5 ml aliquots were treated overnight with a final concentration of 0.02% (w/v) of both nalidixic acid (NA) and yeast extract at 22°C, while rotating at 100 rpm (modified from Kogure et al. 1979). Samples were then stained with 0.01% (w/v) acridine orange (AO) for 2 min at room temperature and collected in 0.2 µm black polycarbonate filters (Millipore), following the indications of Kepner & Pratt (1994). Total and enlarged cell numbers were determined under blue light excitation with a magnification of 1250× using an Olympus BH2-RFC fluorescence microscope. Those cells that were elongated by at least 1.5-fold in length and/or width, with respect to nalidixic acid [NA]-untreated cells, were recorded as NA-active cells (Barcina et al. 1995). A total of 40 fields were counted using a 10 × 10 ocular grid and, subsequently, the mean cell number per field was determined.

INTDC: Cell suspensions were incubated with a final concentration of 0.01% (w/v) of INT for 1 h at 22°C in the dark (modified from Zimmermann et al. 1978). Samples were fixed with a final concentration of 2% (w/v) formaldehyde (FMA) and placed in a Neubauer improved counting chamber. Counts were

performed in duplicate using an Olympus BH2 microscope (1250× magnification). Cells containing an intracellular reddish-brown formazan crystal were recorded as INT-active.

***Plate counts* (*PC*):** Culturable cell numbers were determined by duplicate plating of 0.1 ml aliquots from the microcosms on TSA, and on 20- and 4000-fold dilutions of this medium (TSA 1/20 and 1/4000). These media, except for the freshwater and the 9‰ microcosms, were amended with 1.5 or 2.8% NaCl to mimic the salinity of the 16 or 33‰ microcosms. Plates were incubated for a 3 d period at 22°C and the mean number of cfus was determined. Some of the microcosms were stopped after 5 mo when PC had remained almost constant.

Resuscitation experiments. The resuscitation experiments were started once the culturable cell numbers were fixed at a level below 0.1 cfu ml^{-1}. To attain this level, once PC reached 0 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, TSA 1/20 and TSA 1/4000 (with the appropriate NaCl concentration) and the plates were incubated for 1 wk at 22°C.

In vitro assays: Samples of 1 and 2 ml from the microcosms were added in duplicate to tubes containing 9 or 2 ml (double-strength), respectively, of fresh liquid medium. TSB, and the same medium 20- and 4000-fold diluted (TSB 1/20 and TSB 1/4000), with the appropriate NaCl concentration (as described for TSA), was used. After 1 wk at either 10 or 22°C with rotation, the occurrence of turbidity was recorded as a positive recovery result after both re-isolation on suitable solid media and taxonomic characterization according to Montes et al. (1999). The results were compared to the differential features described for *Vibrio pelagius* (Angulo et al. 1992, Alsina & Blanch 1994).

To find out whether true recovery took place or not, additional resuscitation assays were carried out following the extinction of culturable cells by dilution or treatment with ampicilline at bacteriolytic concentrations (modified from Lleo et al. 1998). In the first procedure, 10-, 100- and 1000-fold dilutions of the microcosms were prepared. The second procedure was the incubation of microcosm aliquots with 150 µg ml^{-1} of ampicilline (except in the control samples). After 24 h at 22°C, 3.37 mg ml^{-1} of penicillinase (0.3 benzylpenicillin units per mg of protein) were added. The working ampicilline concentration was obtained by the broth dilution technique, and tested in the media used to prepare microcosms (3 different salinities and nutrient concentrations). Undiluted, diluted, ampicilline-treated and -untreated samples were all subjected to *in vitro* resuscitation as described above.

In vivo assays: *In vivo* resuscitation was performed by intraperitoneal injection into sea bream weighing 3 g, using 8 fish per challenge. VBNC cells, normal culturable cells (used as positive control) and cell-free nutrient broth with 2% NaCl (NB-2%; used as negative control) were tested in units of 0.1 ml. The aliquots of VBNC cells were taken directly from the microcosms. Normal cultures of Strain 7P obtained in TSA-2% were resuspended in NB-2% to an absorbance of 0.22 at 595 nm (approximately 10^8 cells ml^{-1}). Appropriate dilutions were prepared and the doses, ranging from 2.3×10^4 to 2.3×10^7 cells ml^{-1}, were assayed. The fish were monitored for an 11 d period, after which the internal organs (liver and kidney) of all specimens were sampled. The isolates obtained on TSA-2% were taxonomically confirmed, as indicated in the previous section.

Cell characterization studies. Aliquots were extracted in duplicate from the microcosms once experiments had stopped (in long-term survival microcosms) or once resuscitation assays began (in VBNC microcosms). Samples were stored at –20°C until use. Normal culturable cells were grown in NB-2% for 16 h at 22°C and used for comparison purposes. Samples in units of 50 ml were centrifuged at 10 000 × *g* (Centrikon T-124 centrifuge, Kontron Instruments) for 10 min at 4°C prior to membrane protein extraction and electron microscopy.

Membrane protein profiling (MPP). Total and outer membrane proteins (TMP and OMP, respectively) were extracted following the method of Crosa & Hodges (1982). Cells from 50 ml samples were suspended in 3 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.3% NaCl and sonicated 5 to 7 times for 30 s at 50 W (Branson Sonifier 450) in an ice bath. The unlysed cells were collected at 10 000 × *g* (Sigma 3K-30 centrifuge) for 5 min at 4°C, and the supernatant fraction was either centrifuged at 30 000 × *g* for 1 h at 4°C to sediment the TMP, or treated with 3% (w/v) sodium lauryl sarcosinate prepared in 20 mM Tris-HCl buffer (pH 8.0) for 20 min at room temperature to obtain OMP, which was then harvested at 53 250 × *g* for 1 h at 4°C. TMP and OMP pellets were suspended in 50 µl of distilled water and kept at –20°C until use.

Membrane protein samples, together with a low molecular weight protein standard, were boiled for 10 min in double-strength sample buffer (20% [v/v] glycerol, 4% [w/v] sodium dodecyl sulphate, 10% [w/v] 2-mercaptoethanol, 0.005% [w/v] bromophenol blue). Aliquots containing 2 µg proteins were subjected to electrophoresis on 12.5% (w/v) SDS-PAGE (Laemmli 1970) at 145 V for 50 min.

Gels were fixed in 20% (w/v) trichloroacetic acid for 30 min, and soaked twice in 10% (v/v) ethanol and 5% (v/v) acetic acid for 5 min. They were shaken in 5%

(w/v) glutaraldehyde for 10 min and soaked in the ethanol and acetic-acid solution for a further 10 min, before being rinsed twice in distilled water, oxidized in 0.025% (w/v) sodium dithionite for 1 min, and washed in distilled water. Staining was performed in a silver solution (0.2 g silver nitrate, 75 µl formaldehyde per 100 ml distilled water) for 30 min. After rinsing in distilled water for 1 min, the developing solution was added (4% [w/v] sodium carbonate, 10 µl sodium tiosulphate (5% [w/v]) and 50 µl formaldehyde per 100 ml distilled water). Band developing was stopped with 5% (v/v) acetic acid.

Electron microscopy (EM). Cells from 50 ml samples were suspended in 1 ml PBS and centrifuged at 10 000 × *g* for 10 min at 4°C. Cells were fixed with 2% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer for 2 h at 10°C, washed twice with the buffer and once with 50% (v/v) ethanol. The samples were then placed on cover slips, air-dried and sputter-coated for 2 min. The observations were undertaken using a Philips XL30 microscope. The number of measurements ranged between 25 and 250, according to the availability of cells within the microcosms. Length and width were measured in recorded images using the program Image Tool 2.0. The biovolume was calculated as proposed by Krambeck et al. (1981).

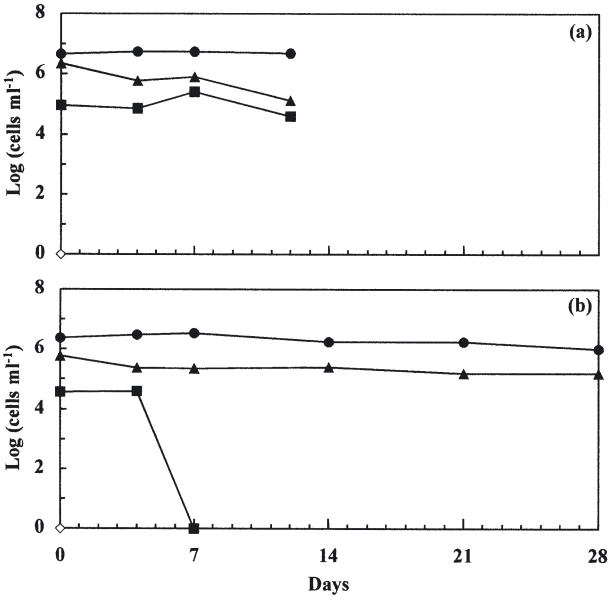

Fig. 1. *Listonella pelagia.* Survival of Strain 7P in fresh water at (a) 4 and (b) 22°C. ●: acridine orange total counts (AOTC), ■: nalidixic acid direct counts (NADC), ▲: INT direct counts (INTDC), ◇: plate counts (PC). Each value represents the mean from 2 determinations. PC values are means from determinations in 3 solid media (TSA and 20- and 4000-fold diluted TSA). Data are representative of duplicate survival experiments with SD below 12%

Statistical analysis. Statistical analysis was performed using the program SPSS 10.0. Counting procedures were compared 2 by 2 using 1-way ANOVA, including incubation temperature, salinity and nutrient content as co-variables. The influence of these variables on the survival time of the strain was evaluated by regression analysis. The length, width, width:length ratio and biovolume of normal culturable cells and cells from the microcosms were also compared by ANOVA. Probability values equal to or below 0.05 were considered to be statistically significant.

Culture media and chemicals. Culture media or their components were purchased from Cultimed. Ampicilline was obtained from Roche. Tricine, AO, NA, INT, penicillinase, sodium lauryl sarcosinate, glycerol, sodium dodecyl sulphate, mercaptoethanol, bromophenol blue, sodium dithionite and silver nitrate were obtained from Sigma. The protein standard was obtained from BioRad. Other chemicals were analytical grade and purchased from Panreac.

RESULTS

Analysis of the counting procedures

The comparison of the different counting procedures revealed that the number of cfu obtained on TSA, TSA 1/20 and TSA 1/4000 was statistically identical. However, when AOTC, INTDC, NADC and PC (the average value obtained for the 3 solid media employed) were compared 2 by 2, the results were statistically different. AOTC gave the highest yields, followed by INTDC, NADC and PC.

Survival of *Listonella pelagia* in natural freshwater and defined-medium microcosms

As Strain 7P displayed homogeneous behaviour in the microcosm duplicates (with overall standard deviations below 12%), one of these samples was chosen for graphical representation. When cells of *Listonella pelagia* were cultured in fresh water, the survival time was 0 d and a VBNC response could be observed (Fig. 1a). After colonies could no longer be detected on any solid media, continued cell activity was revealed by the 2 direct assays employed, except in fresh water incubated at 22°C, where NADC dropped below the limits of detection at Day 7 and only respiratory activity was subsequently maintained (Fig. 1b). Table 1 reflects the average survival times of Strain 7P in the completed defined medium microcosms. The values ranged from 0 to more than 439 d.

Table 1. *Listonella pelagia.* Survival time (days, mean value from microcosm duplicates and 2 determinations on the solid media employed) of Strain 7P in the defined media. Microcosms are referred to by their incubation temperature (°C), salinity (‰) and nutrient concentration (g l^{-1}). Those microcosms that were sampled for membrane protein profiling (MPP) and electron microscopy (EM) are indicated. The width-to-length ratio is provided (mean ± SD) for those cases where changes were statistically significant ($p < 0.05$) and a coccoid morphology was detected. nt: not tested, nf: not found

Microcosm °C	‰	g l^{-1}	Survival time (d)	Sample	Width:length (mean ± SD)
4	9	0.005	0	MPP, EM	0.59 ± 0.18
4	9	1	5	MPP	nt
4	9	17	0	MPP	nt
4	33	0.005	37	MPP, EM	nf
4	33	1	314[a]	nt	nt
4	33	17	21	nt	nt
10	9	0.005	1	MPP	nt
10	9	1	1	MPP	nt
10	9	17	10.33	nt	nt
10	16	1	42	MPP, EM	0.70 ± 0.16
10	33	0.005	439[a]	MPP, EM	0.60 ± 0.25[b]
10	33	1	314[a]	MPP, EM	0.75 ± 0.16
10	33	17	20	nt	nt
22	33	0.005	153[a]	MPP, EM	0.82 ± 0.17
22	33	1	189[a]	nt	nt
22	33	17	15	nt	nt

[a]Time when counts were stopped in the long-term culturable microcosms
[b]$p = 0.087$

PC values dropped to zero at the start of the experiments or soon after inoculation (Fig. 2), at all of the nutrient concentrations assayed for the defined medium of 9‰ incubated at 4 or 10°C, with a registered maximum survival time of 10.33 d (Table 1). NADC decreased below the limits of detection at this time, or after culturability was lost when only INT-active cells were present. However, cells inoculated into 16‰ medium (1 g l^{-1} of nutrient content and cultured at 10°C) demonstrated a longer survival ability (42 d). Thereafter, the values for AOTC, NADC and INTDC remained constant (Fig. 3).

When the defined medium of 33‰ and 17 g l^{-1} nutrient content was tested, cells became non-culturable and the activities measured by NA and INT assays were maintained (at 4°C), showed a slight decrease (at 10°C, Fig. 4c) or dropped to below detection levels (at 22°C). However, when lower nutrient concentrations (0.005 or 1 g l^{-1}) were assayed (at 33‰), cells, both culturable and active, survived for more than 153 d (Table 1, Fig. 4a,b). In these cases, a slight decrease both in direct counts and PC was observed as the survival time increased, suggesting that part of the population was being lysed. The only exception to this behaviour was the microcosm cultured at 4°C with 0.005 g l^{-1} of nutrient content (graph not shown), where cells entered a VBNC state. NADC fell below the detection limits 20 d later than PC, showing a similar pattern to that of the microcosm represented in Fig. 2.

In short, 2 survival responses were detected during the experimental period: either cells remained culturable and active for long periods (long-term survival) or they entered a VBNC state in which subpopulations differing in their activities were formed.

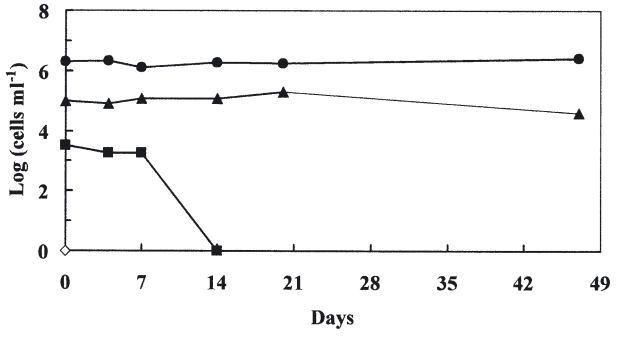

Fig. 2. *Listonella pelagia.* Survival of Strain 7P in 9‰ and 0.005 g l^{-1} (nutrient content) defined-medium microcosm at 4°C. ●: AOTC, ■: NADC, ▲: INTDC, ◇: PC. (See Fig. 1 legend for abbreviations.) Each value represents the mean from 2 determinations. PC values are means from determinations in 3 solid media (TSA and 20- and 4000-fold diluted TSA). Data are representative of duplicate survival experiments with SD below 12%

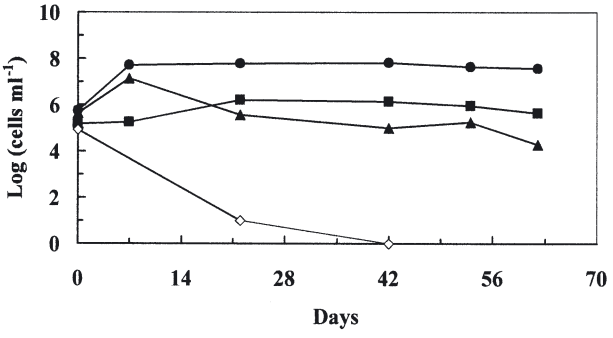

Fig. 3. *Listonella pelagia.* Survival of Strain 7P in 16‰ and 1 g l^{-1} (nutrient content) defined-medium microcosm at 10°C. ●: AOTC, ■: NADC, ▲: INTDC, ◇: PC. (See Fig. 1 legend for abbreviations.) Each value represents the mean from 2 determinations. PC values are means from determinations in 3 solid media (TSA, and 20- and 4000-fold diluted TSA). Data are representative of duplicate survival experiments with SD below 12%

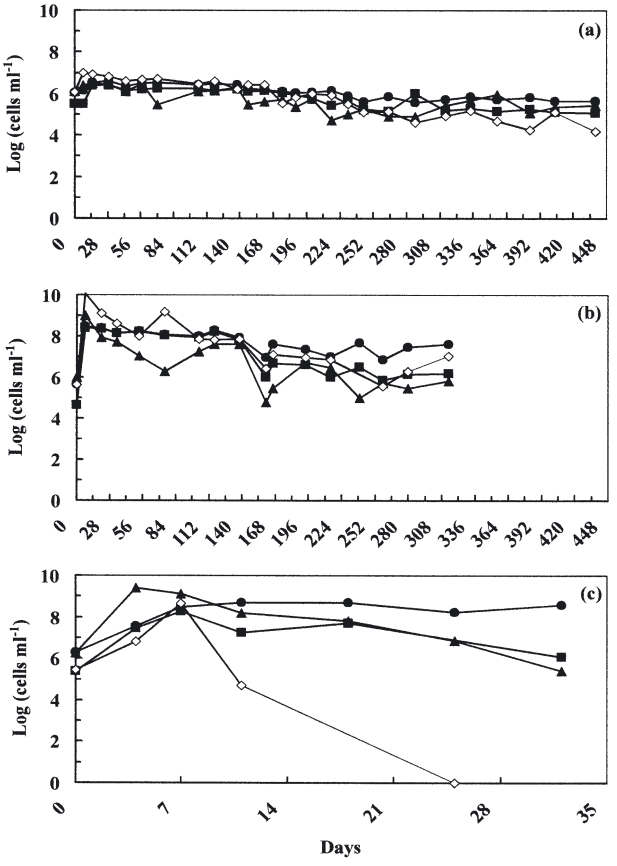

Fig. 4. *Listonella pelagia.* Survival of Strain 7P in 33‰ and (a) 0.005, (b) 1 and (c) 17 g l^{-1} (nutrient content) defined-medium microcosms at 10°C. ●: AOTC, ■: NADC, ▲: INTDC, ◇: PC. (See Fig. 1 legend for abbreviations.) Each value represents the mean from 2 determinations. PC values are means from determinations in 3 solid media (TSA, and 20- and 4000-fold diluted TSA). Data are representative of duplicate survival experiments with SD below 12%

However, it was observed that the temperatures studied did not affect the persistence of *Listonella pelagia* in laboratory microcosms. In fact, the equation obtained by regression analysis confirmed that both the nutrient concentration and salinity, but not the temperatures assayed, were determinants of the average survival time of *L. pelagia* in the defined medium microcosms:

$$T_{surv} = k + 7.0 \times S - 7.6 \times N \quad (R^2 = 0.51)$$

where T_{surv} (d) is the mean survival time from microcosm duplicates and 2 determinations on the 3 solid media employed, S is the salinity (‰), N is the nutrient concentration (g l^{-1}), k is the equation constant and R^2 the regression coefficient.

In vitro resuscitation

In 4 of the 13 cases where a VBNC state was detected, *in vitro* resuscitation of 1 or 2 ml undiluted samples was found at 22°C. When assays were performed at 10 and 22°C (see Table 2), in 1 case recovery was only obtained at the higher temperature. Recovery was only detected in TSB medium.

Additional resuscitation assays were performed on these microcosms in the same conditions as previously employed, using up to a 1000-fold dilution (where less than 0.0001 culturable cells ml^{-1} were present) and treatment with ampicilline at bacteriolytic concentrations. The concentration chosen (150 µg ml^{-1}) was able to inhibit 10^6 cfu ml^{-1} after incubation for 24 h at 22°C, irrespective of the defined medium used. Only in one of the microcosms (10°C, 33‰ and 17 g l^{-1} nutrient concentration) was true resuscitation confirmed by both dilution and ampicilline assays, as shown in Table 2. The isolates obtained on TSA-2% from positive tubes displayed the differential features described for *Vibrio pelagius*: Gram-negative, oxidase-positive, glucose-fermenting, growth on TCBS and at different NaCl concentrations (0, 4 and 6%), arginine dihydrolase-negative, ornithine and lysine decarboxylase-negative, indole-positive, gelatinase-positive, arabinose-positive and sacarose acid-positive.

In vivo resuscitation

Injection of both culturable (positive control) and VBNC cells from 1 out of the 4 microcosms assayed produced haemorrhages in the mouth, base of fins and

Table 2. *Listonella pelagia. In vitro* resuscitation of undiluted (1), diluted (1/10, 1/100 and 1/1000), ampicilline-treated and -untreated samples. Microcosms are referred to by their incubation temperature (°C), salinity (‰) and nutrient concentration (g l^{-1}). Each experiment was performed in duplicate using 2 replicate tubes. Results are shown for the 3 liquid media used (TSB, 20- and 4000-fold diluted TSB, all amended with NaCl to mimic the salinity of the microcosms) and the different sample volumes inoculated (1 or 2 ml), unless otherwise indicated. +: visible turbidity in the tubes and growth on solid media, –: neither visible turbidity nor growth on solid media

Microcosm			Undiluted	Diluted			Ampicilline	
°C	‰	g l^{-1}	1	1/10	1/100	1/1000	Untreated	Treated
4	9	1	+	–	–	–	+	–
10	9	1	+	–	–	–	+	–
10	9	17	+	–	–	–	+	–
10	33	17[a]	+[b]	+[b]	+[b]	+[b]	+[b]	+[b]

[a]Resuscitation at 10°C not successful
[b]In TSB

Table 3. *Listonella pelagia*. *In vivo* and *in vitro* resuscitation. Microcosms are referred to by their incubation temperature (°C), salinity (‰) and nutrient concentration (g l^{-1}). NADC: nalidixic acid direct counts, INTDC: INT direct counts, DL: detection limits (8×10^4 cells ml^{-1} for NADC and 2×10^4 cells ml^{-1} for INT), +: visible turbidity in the tubes and growth on solid media (*in vitro* assays), or symptoms in fish and re-isolation from internal organs of sea bream (*in vivo* assays), –: neither visible turbidity nor growth on solid media (*in vitro* assays), or neither symptoms in fish nor re-isolation on solid media *in vivo* assays. nt: not tested. For the resuscitation assay, time was measured as days elapsed since the last NADC and INTDC

Microcosm			Cell count		Resuscitation assay		
°C	‰	g l^{-1}	NADC	INTDC	Time (d)	*In vitro*	*In vivo*
4	9	1	<DL	6.5×10^4	8	–	–
4	33	17	2.7×10^6	7.2×10^6	2	–	+
10	9	1	<DL	3.3×10^4	1	–	nt
10	9	17	<DL	3.8×10^5	0	–	–
10	33	17	1.2×10^6	3.5×10^6	2	+	–

internal organs of fish, and a pale liver with no apparent necrotic areas (see Table 3). Mortalities were only recorded in fish used as positive controls. Strain 7P was re-isolated from the liver and kidney of all dead or symptomatic fish, and showed the differential features described in the previous section. No isolates were obtained from the internal organs of sea bream that were used as negative controls.

Study of the membrane protein profiles

No changes were detected in the membrane protein profiles of cells from the 9 microcosms analysed (Table 1), as compared to those of normal culturable cells. However, a 36 kDa protein band proved to be slightly intensified in the total- and outer-membrane protein profiles of the defined medium microcosm of 33‰, 0.005 g l^{-1} nutrient concentration, and 10°C incubation, where cells remained culturable after 439 d.

Changes in cell size and shape

The normal culturable cells of Strain 7P were rod-shaped, with average dimensions (mean ± -SD) of 0.55 ± 0.31 µm width by 1.39 × 0.99 µm length (width:length ratio of 0.43:0.10). In 4 out of the 6 microcosms studied (2 of the 3 long-term survival microcosms, and 2 of the 3 showing a VBNC response), cells turned coccoid as shown by a width:length ratio statistically different and closer to 1 than that of culturable cells. In the third long-term culturable microcosm, this ratio was significantly different ($p = 0.087$; Table 1). However, for the first 2 long-term culturable microcosms, cells diminished their biovolume either by 72 or 82% (cells surviving for 153 and 314 d, respectively). However, an increase of 111% in the biovolume was observed in the 16‰ VBNC microcosms.

DISCUSSION

Two survival strategies for *Listonella pelagia* are described in this study. Cells remained culturable for periods ranging from >5 mo to >1 yr in the defined media that had salinity and nutrient concentration values closer to those of the marine environment. Survival times of >1 yr have been recorded for *Vibrio salmonicida* (Hoff 1989) and *V. vulnificus* (Biosca et al. 1996) kept in artificial or natural seawater. However, a VBNC state was detected at the same salinities of estuarine, or freshwater areas (as described by Eguchi et al. 2000), or when highly nutritive defined media were used.

After the cells of *Listonella pelagia* failed to form colonies, the populations became heterogeneous, in terms of the activities revealed by nalidixic acid and INT-reduction assays. Although the limits of detection demand the presence of a minimum number of active cells, the different subpopulations detected confirm the relevance of these techniques for the study of the VBNC state (Rahman et al. 1994, Huq & Colwell 1995). Under certain conditions, the fall of the NADC level below the limits of detection indicates that at least part of the population has lost the ability to uptake substrate. Despite this, respiratory activity was maintained. Consequently, subpopulations found for each microcosm were different and the complete death sequence described for starved *Salmonella typhimurium* (Joux et al. 1997) was not observed during the experimental period for *L. pelagia*.

To ensure the occurrence of a true recovery and to preclude the possibility of regrowth, resuscitation assays were performed in some of the microcosms using up to a 1000-fold dilution (where less than 0.0001 culturable cells ml^{-1} would be present), and treatment with ampicilline was carried out at concentrations that would kill actively growing (i.e. culturable) cells. Ravel et al. (1995) demonstrated that recovery in diluted samples containing only 0.085 cfu ml^{-1} was due to the regrowth of culturable cells. However, in our case a true resuscitation was found in one of the microcosms assayed. Similar results have been reported for *Enterococcus faecalis* (Lleo et al. 1998). The results suggest incubation temperature has an influence (as reported by Biosca et al. 1996), as any increase was determinant

on the recovery of VBNC cells. Also, the nutrient concentration of the resuscitation media played a role in this response. The fact that resuscitation was not achieved in all cases indicates that the conditions involved in the exit from the VBNC state are different for each experiment (McDougald et al. 1998), and consequently a positive response in the natural environment cannot be discounted (Weichart et al. 1992).

For *in vivo* assays (less than 0.01 cfu inoculated into fish), VBNC cells of *Listonella pelagia* are able to show infectivity and recover culturability after passage through fish. Reproducibility of symptoms would confirm the role of VBNC cells of *L. pelagia* in disease outbreaks. Similar results have been found for species such as *Pasteurella piscicida* (Magariños et al. 1994) and *Vibrio vulnificus* biotype 2 (Marco-Noales et al. 1999).

It appears from our results that the existence of a population with respiratory activity and the ability to uptake substrate is necessary, but not entirely sufficient, to ensure *in vitro* or *in vivo* recovery. For example, injection into fish did not always lead to improved recovery conditions, a finding which is contrary to that proposed by Mukamolova et al. (1998).

No changes in the membrane protein profiles of the VBNC cells of *Listonella pelagia* were detected (the same was found for *Pasteurella piscicida* by Magariños et al. 1994 and for *Aeromonas salmonicida* by Effendi & Austin 1995), whereas a protein band of 36 kDa was slightly intensified in the cell populations surviving 439 d. This indicates a lack of specific membrane protein synthesis during the VBNC process. Moreover, new proteins were found to be present in the outer membrane of the long-term culturable Strain S14 of *Vibrio* sp. (Kjelleberg et al. 1993).

Examination of cells under EM revealed a spheroid morphology. These changes are in agreement with those reported for *Vibrio vulnificus* (Oliver, 2000) and starved culturable *Yersinia ruckeri* (Thorsen et al. 1992), and are assumed to be a survival strategy to optimize substrate uptake by means of an increased surface-to-volume ratio (Lázaro et al. 1999). The reduction in size and biovolume found for long-term surviving cells are in agreement with the changes found for species like *V. cholerae* (Wai et al. 1999) and *Photobacterium damselae* ssp. *damselae* (Fouz et al. 1998). However, the increase in biovolume found for VBNC cells of *Listonella pelagia* cultured in a 16‰ medium, and the spheroid morphology, would suggest that although membrane activity is maintained, the cells are under osmotic stress implying an alteration of the cell wall, as reported by Signoretto et al. (2000). Yet in our case it should be pointed out that changes in cell shape and size could not be related to changes in the membrane protein profiles.

In this study the survival strategies of *Listonella pelagia* in fresh water and different defined media have been described. Both long-term survival and a VBNC state have been observed, confirming the ability of *L. pelagia* to adapt itself to a changing environment. Morphological and size changes were found in long-term culturable and VBNC cells. In at least 1 case the recovery of VBNC cells was confirmed by 2 independent assays. The VBNC cells of *L. pelagia* were recoverable and infective, thus confirming its behaviour as an opportunistic pathogen under certain environmental conditions, indicating potential risk towards the health of aquatic organisms.

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