RESEARCH ARTICLE

Characterization and virulence retention of viable but nonculturable Vibrio harveyi

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Abstract
Vibrio harveyi has been reported to enter into a viable but nonculturable (VBNC) state. Two clinical V. harveyi strains, SF1 and CW2, and the type strain, VIB295 T, were incubated in sterilized seawater at 4 °C. Plate counts in these strains declined to undetectable levels (< 0.1 CFU mL⁻¹) within 69, 67 and 65 days, respectively. The direct viable count (DVC) declined from 10⁶ to 10⁴ active cells mL⁻¹ and remained constant at this level by a DVC. VBNC cell numbers could be restored via a temperature upshift when grown in yeast extract with the addition of Tween 20 or compound vitamin B. Reverse transcriptase-PCR was used to monitor virulence gene expression within VBNC cells. No expression of the hemolysin gene was detected in VBNC cells. VBNC and resuscitative cells were intraperitoneally injected into zebra fish separately. No death was observed in the groups inoculated with VBNC cells. The fish inoculated with the resuscitative cells died within 7 days, the lethal dose 50% (LD₅₀) being 2.85 × 10⁴ CFU mL⁻¹, a value similar to that for groups inoculated with normal cells (2.28 × 10⁴ CFU mL⁻¹). This suggested that VBNC V. harveyi might retain pathogenic potential.

Introduction
Vibrio harveyi is a Gram-negative, luminous, marine bacterium occurring either free living or in association with marine forms of life such as fish and crustaceans (O’Brien & Sizemore, 1979; Orndorff & Colwell, 1980; Ramesh & Venugopalan, 1989). The organism has been reported as a major causal agent of luminous vibriosis, which affects a diverse range of marine invertebrates. Mass mortalities of shrimp larvae due to V. harveyi infection have been reported from a number of countries, especially in South America and south-east Asia (Liu et al., 1996; Alvarez et al., 1998; Vandenbergh et al., 1998). Additionally, V. harveyi has been associated with diseases in fish (Sunaryanto & Mariam, 1986; Saeed, 1995; Hispano et al., 1997). The pathogenicity of V. harveyi can be attributed to the presence of extracellular virulence factors such as proteases, hemolysins, chitinases, lipases and phospholipases (Liu et al., 1996). Vibrio harveyi luminescence has been related to protease toxin activity (Nakayama et al., 2006). Quorum sensing in V. harveyi has also been reported to be related to regulation of virulence genes (Lowery et al., 2005). However, the mechanisms of pathogenicity of V. harveyi are not completely understood and the causes of disease occurrence remain elusive.

It has been reported that many Vibrio species can enter into the viable but nonculturable (VBNC) state as a response to natural stresses (Oliver & Bockian, 1995). The characterization of these pathogenic bacteria in the VBNC state has been well documented (Xu et al., 1982; Oliver & Wanucha, 1989; Wong et al., 2004). Cells entering the VBNC state change from short rods to coccoids and decrease in size, and some metabolic changes occur, including reductions in nutrient transport, respiration rates and macromolecular synthesis (Porter et al., 1995; Oliver, 2000). It has also been reported that under appropriate conditions these microorganisms can nevertheless recover from this dormant state, becoming metabolically active and fully culturable (Roth et al., 1988; Nilsson et al., 1991; Whitesides & Oliver, 1997; Mizunoe et al., 2000). Some pathogenic bacteria may retain pathogenicity when in the VBNC state (Colwell et al., 1985; Oliver & Bockian, 1995; Baffone et al., 2003). Recent studies have demonstrated gene expression by cells in the VBNC state (Lleó et al., 2000; Yaron & Matthews, 2002). Saux & Dominique (2002) reported continued production of mRNA for several genes of Vibrio vulnificus for as long as...
4.5 months after the cells entered into the VBNC state. Ramaiah et al. (2002) reported that luminous V. harveyi ATCC 14126 could enter the VBNC state in response to nutrient-limited and low-salinity incubation at 22 °C; recovery of culturability of cells in the VBNC state was achieved by addition of nutrient broth (Ramaiah et al., 2002).

The aim of the present study was to characterize the experimentally induced VBNC forms of clinical V. harveyi isolates and their virulence retention.

Materials and methods

Bacterial strain and growth conditions

*Vibrio harveyi* VIB295T (LMG4044T) was obtained from the bacteria collection in the School of Life Sciences (Heriot-Watt University, Edinburgh, UK). Strains SF1 and CW2 were isolated from diseased seabass (*Lateolabrax japonicus*) and turbot (*Scophthalmus maximus*) in China and were identified as representing *V. harveyi* by the Vitek 32 microbial analysis system (bioMérieux, Marcy l’Etoile, France) and 16S rRNA gene sequence analysis. Authenticity was then verified based on Holt et al. (1994). The strains were stocked in cultured broth with 10% (v/v) glycerol at −85 °C, and cultured in 2216E agar at 26 °C.

Preparation of microcosms of *V. harveyi*

Microcosms were prepared by filtering seawater through a 0.22-μm pore-size filter (Millipore) and sterilized by autoclaving. *Vibrio harveyi* cells were cultured in 2216E agar overnight at 26 °C to the logarithmic phase of growth, and the cells were washed with sterile 0.22-μm pore-size filtered seawater. The washed cells were then inoculated into the seawater microcosms at a final concentration of c. 10⁶ cells mL⁻¹ and maintained at 4 °C without shaking to induce the VBNC state as described (Lleò et al., 2000; Du et al., 2007).

Culturability and viability assays

Culturability was determined every 6 days via a spread plate count (PC) with 2216E agar plates. Samples from the microcosms were serially diluted in sterilized seawater, spread in triplicate on 2216E agar and incubated at 26 °C for 48 h. When the culturable cell populations were < 10 CFU mL⁻¹, a 10-ML aliquot of the microcosm was filtered onto a 0.22-μm pore-size filter and the filter placed on 2216E agar plates. The bacteria were considered to be nonculturable when < 0.1 CFU mL⁻¹ of the culturable cells could be detected in the microcosms (Baffone et al., 2003).

To determine the total number of cells, samples from the microcosm were serially diluted, fixed with formalin (2%, v/v), stained with acridine orange (0.01%, w/v) for 2 min, and filtered onto 0.22-μm pore-size black polycarbonate filters (Millipore). The filters were then examined under an Olympus BH-2 epifluorescence microscope (Japan).

The number of viable cells was measured by a direct viable count (DVC) method, as described by Kogure et al. (1979). Yeast extract (0.025%, w/v) and nalidixic acid (0.002%, w/v) were added to the serially diluted samples, the samples were incubated at 26 °C for 14 h, cells were stained with acridine orange and observed as described above. Viable cells that responded to this treatment in the presence of yeast extract and nalidixic acid became elongated to at least twice the length of cells treated with acridine orange direct count (AODC).

Each method was tested in three replicates with reproducible results. Statistical analysis was performed using the commercial software program spss10.0.

Resuscitation of VBNC cells

For resuscitation of VBNC cells with increasing temperature, 5 mL of the VBNC cells was removed from the microcosms and allowed to incubate at 26 °C with or without addition of yeast extract (0.025%, w/v). Culturability was determined by plating 0.1-mL samples on 2216E agar.

To determine the effects of different chemicals on resuscitation of the VBNC *V. harveyi*, Tween 20 and compound vitamin B were added to samples of the VBNC cells containing yeast extract at final concentrations of 6% (v/v) and 1 mg mL⁻¹, respectively. The samples were then incubated at 26 °C for 48 h to determine culturability. All experiments were performed in triplicate.

Scanning electron microscopy

*Vibrio harveyi* cells in different states were fixed with 3% (v/v) glutaraldehyde at room temperature for 4 h, and filtered onto 0.22-μm pore-size nucleopore polycarbonate filters. The samples were then dehydrated and coated as described by Du et al. (2007). The coated samples were then observed under a JSM-840 scanning electron microscope (JEOL, Japan).

Reverse transcriptase (RT)-PCR detection of the virulence gene of VBNC cells

Total RNA extraction

For the culturable samples, 5 mL of cells at the logarithmic phase were centrifuged at 11200 g for 5 min at 4 °C and the cell pellets were collected. Cells in the VBNC state (10⁴ active cells mL⁻¹) were harvested by aseptic filtration (25-mL volumes) using a 25-μm-diameter autoclaved polycarbonate filter (0.2-μm pore-size Nuclepore) placed on a Millipore membrane. The polycarbonate filter was rolled up and transferred, using sterile forceps, into a 1.5-mL
polypropylene tube containing 1 mL of TRIzol reagent; 0.2 mL of chloroform was added to the TRIzol Reagent. The tubes were vortexed vigorously for 30 s and incubated for 2 min, then centrifuged at 10 000 r.p.m. for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube, 0.5 mL of isopropyl alcohol was added to the tube containing 1 mL of TRIzol reagent and incubated for 10 min, then centrifuged at 10 000 r.p.m. for 15 min at 4 °C. The RNA pellets were washed twice with 1 mL of 70% ethanol and dissolved at 25 μL of diethylpyrocarbonate-treated water and stored at −80 °C.

Oligonucleotide primers

The two primers (forward, 5'-TCACCTCTTTGCAGCC GATAACC-3'; reverse, 5'-CAAGGCTTCGCGTA GTCT GATTT-3') delineate a 455-bp region within the ORF of the hemolysin gene unique to V. harveyi.

RT-PCR amplification

DNase-treated RNA (4 μL) was reverse transcribed in a 20-μL volume with 5 mM MgCl₂, 1 × PCR buffer II [50 mM KCl, 10 mM Tris-HCl (pH 8.3)], 1 mM each dNTP, 1 U of RNase inhibitor per microliter, 2.5 U of murine leukemia virus reverse transcriptase per microliter, and 1 μM reverse primer. The tubes were incubated at 42 °C for 15 min, heated at 96 °C for 5 min and cooled to 5 °C for at least 5 min. Half of the RT mixture was added to 40 μL of the PCR mixture. The final concentrations in the PCR mixture were as follows: 2 mM MgCl₂, 1 × PCR buffer II, 200 μM each dNTP, 1.25 U of AmpliTaq DNA polymerase per 50 μL, and 0.5 μM each primer. The thermocycling program was optimized as follows: a first denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After a final extension at 72 °C for 10 min, the tubes were cooled to 4 °C.

Virulence of VBNC and resuscitative cells

Zebra fish were used to investigate the virulence retention of VBNC and resuscitative cells. One-hundred zebra fish were divided into 10 groups, each comprising 10 fish. The fish were kept in 2-L tanks with aerated water at 16–20 °C and fed with commercial pellets. The 10 groups were injected intraperitoneally with 20 μL of the VBNC cells (10⁸ CFU mL⁻¹), resuscitative cells (10⁶, 10⁵, 10⁴ and 10³ CFU mL⁻¹), normal cells (10⁷, 10⁶ and 10⁵ CFU mL⁻¹) and autoclaved saline respectively. The injected fish were then kept at 16–20 °C for 30 days and the mortality was recorded. The lethal dose 50% (LD₅₀) was calculated by the method of Reed & Muench (1938).

Biochemical characterization of resuscitative cells – API 20E test

Biochemical characterization of resuscitative cells of V. harveyi strains SF1 and CW2 was made by API 20E test strips (API system, Montalieu-Vercieu, France) according to the manufacturer’s instructions.

Results

Entry into the VBNC state of V. harveyi

Figure 1 shows the changes in cell numbers of V. harveyi VIB295⁷, SF1 and CW2 during incubation in seawater microcosms at 4 °C. Total cell counts remained constant over 80 days, but PCs rapidly declined from 10⁶ CFU mL⁻¹ to undetectable levels (< 0.1 CFU mL⁻¹) by 65, 69 and 67 days, respectively. Active cells, by contrast, declined to c. 10⁵ CFU mL⁻¹ and remained fairly constant at this level, as demonstrated by DVC staining. These results indicated that a large population of cells existed in the VBNC state.

Resuscitation of VBNC cells

When the VBNC cells of V. harveyi were subjected to a temperature increase (from 4 to 26 °C) in the presence or absence of yeast extract, no culturable population was observed on 2216E agar. When the VBNC cell solutions containing yeast extract were supplemented by Tween 20 or compound vitamin B and subjected to a temperature increase (from 4 to 26 °C), cell numbers were restored. The culturable cell counts rose to about 10⁶ CFU mL⁻¹ on days 8 and 9 on 2216E agar (Fig. 2).

Morphological changes of VBNC V. harveyi cells

The VBNC cells exhibited several morphological changes under the epifluorescence microscope via the AODC method. Compared with normal cells, the VBNC cells changed from short rods to coccoid cells and decreased in size. Red, elongated cells were observed via DVC (not shown). Similar changes could be observed under the scanning electron microscope (data not shown). The average size of the normal cells was 1.5 × 0.5 ± 0.1 μm, but the average radius of the coccoid VBNC cells was 0.2 ± 0.1 μm. The normal cells and restored cells showed no morphological difference.

Detection of mRNA for the hemolysin gene in VBNC V. harveyi cells

We attempted to monitor expression of the virulence gene from the VBNC cultures by RT-PCR. The mRNA for the hemolysin gene was amplified in the normal cells and resuscitative cells, but expression of the gene was not detectable in the VBNC cells (Fig. 3).
Virulence of the VBNC and the resuscitative cells

Some of the zebra fish in groups inoculated with normal and resuscitative *V. harveyi* died over a period of 7 days. LD$_{50}$ for the resuscitative cells was $2.85 \times 10^4$ CFU mL$^{-1}$, which was close to that for normal cells ($2.28 \times 10^4$ CFU mL$^{-1}$). The dead fish all showed similar symptoms, such as ascites and congestion of the focus. Culturable cells of *V. harveyi* could be isolated from ascites fluid and organs. The bacteria were identified by PCR amplification and analysis of the 16S rRNA gene. The fish inoculated with the VBNC cells and autoclaved saline remained alive during the experimental period, and *V. harveyi* was not isolated from these animals.

Discussion

A number of bacteria have been reported to be capable of entering into a VBNC state as a response to environmental stresses, such as starvation, incubation outside the temperature range of growth and elevated osmotic concentrations (Oliver, 2005; Chattopadhyay, 2006). The time entering into such state varies greatly in different bacteria and their physiological state. Wong et al. (2004) reported that 24 strains of *Vibrio parahaemolyticus* from clinical and environmental sources entered the VBNC state in 14–49 days when incubated in artificial seawater at 4.5°C. *Vibrio vulnificus* CVD713 and C7184 entered the VBNC state in artificial seawater at 5°C within 7 days (Oliver & Bockian, 1995), while Linder & Oliver (1989) reported that *V. vulnificus* entered the VBNC state after incubation for 24 days.

Duncan et al. (1994) observed that *V. harveyi* 1280 entered into the VBNC state within 11 days at 4°C. Ramaiah et al. (2002) suggested that the adaptation of *V. harveyi* ATCC 14126 to the VBNC state was influenced by salinity.
In the present study, we used two clinical *V. harveyi* strains, SF1 and CW2, and the type strain, VIB295T. These three strains became nonculturable when inoculated in seawater microcosms at 4°C within 65–69 days. These data were in agreement with those of *V. harveyi* ATCC 14126 incubated in microcosms of 35g kg⁻¹ artificial seawater at 22°C (Ramaiah et al., 2002).

An important characteristic of the VBNC cells is their ability to be resuscitated *in vitro* and in natural estuarine environments (Oliver & Bockian, 1995). Resuscitation of the VBNC cells was achieved by a temperature increase in the presence of yeast extract by addition of either Tween 20 or compound vitamin B, but their exact mechanisms in this process remain to be clarified. Ramaiah and Ravel reported that *V. harveyi* ATCC 14126 cell numbers could be restored through a temperature increase without nutrients, and they also obtained an increase in luminescence and cell length (indicating viability) on an increase from 4 to 30°C. In the present study, a temperature increase with and without the presence of nutrition did not result in recovery from nonculturability.

When entering into the VBNC state, *V. harveyi* cells decreased in size and became coccoidal, which was in agreement with the results reported by other authors (Oliver & Wanucha, 1989; Morita, 1993; Benjamin & Datta, 1995; Federighi et al., 1998). Several studies have demonstrated that the VBNC cells showed greater resistance to various stress conditions (Jiang & Chai, 1996; Chaiyanan et al.,...
2001). There were no significant differences in antibiotic resistance and main biochemical characterizations between the restored cells and normal cells of *V. harveyi* SF1 and CW2 (data not shown).

It has been reported that some pathogens are able to express the virulence determinant and toxin genes in the VBNC state. *Vibrio cholerae* showed a different reduction in the number of copies of 16S rRNA and mRNA for the tuf, rpoS and relA genes, but the mRNA for the relA gene was selectively increased in VBNC cells (González-Escalona et al., 2006). Lleò et al. (2000) reported the expression of a gene involved in peptidoglycan synthesis in VBNC forms of *Enterococcus faecalis*. Cytotoxin–hemolysin mRNA was detected in VBNC populations of environmental and clinical *V. vulnificus* strains maintained in artificial seawater (Saux & Dominique, 2002). Coutard et al. (2005) reported that the 16S–23S rRNA and rpoS genes of *V. parahaemolyticus* Vp4 were expressed in VBNC cells whereas no expression of the *tdh1* and *tdh2* genes were observed in the same populations. We could not detect hemolysin mRNA of the VBNC cells of *V. harveyi* by RT-PCR, but the mRNA for the 16S rRNA gene was detectable in the same VBNC cells. The mRNA for the hemolysin gene could be detected in the normal and resuscitative cells.

It has been known that some *Vibrio* species can initiate infections from the VBNC state. *Vibrio cholerae* cells in the VBNC state were recovered from rabbit ileal loops in which enterotoxigenicity was exhibited (Kogure et al., 1979; Duncan et al., 1994). Baffone et al. (2003) reported that VBNC *V. parahaemolyticus* and *Vibrio alginolyticus* strains lost the virulence characteristics after resuscitation in mouse and were subsequently reactivated by means of two consecutive passages of the strains in a rat ileal loop model. Fish injected with the VBNC *V. harveyi* SF1 remained alive and did not show any visible signs. The resuscitative *V. harveyi* SF1 cells showed pathogenicity when they were intraperitoneally inoculated into fish. The fish inoculated with the resuscitative cells died within 7 days, showing similar symptoms with those inoculated with the normal cells. *Vibrio harveyi* was isolated from these organs. This result was in agreement with the analysis of hemolysin mRNA in the VBNC cells, normal cells and resuscitative cells. Because hemolysin has been reported to be the major virulence factor of *V. harveyi* (Sun et al., 2007), it could be assumed that the loss of the pathogenicity in VBNC cells of *V. harveyi* SF1 was due to a temporary inability to express its virulence characteristics, such as hemolysin genes. The resuscitative *V. harveyi* SF1 cells expressed the hemolysin gene and showed pathogenicity to the fish. These results indicated that VBNC *V. harveyi* might retain its pathogenic potential and cause disease.

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**References**


Coutard F, Pommepuy M, Loaec S & Hervio-Heath D (2005) mRNA detection by reverse transcription-PCR for monitoring viability and potential virulence in a pathogenic strain of...


