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mRNA detection by reverse transcription–PCR for monitoring viability and potential virulence in a pathogenic strain of Vibrio parahaemolyticus in viable but nonculturable state

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Abstract: Aims: This work investigates the maintenance of viability and potential virulence of Vibrio parahaemolyticus in a viable but nonculturable population (VBNC) state by reverse transcription–polymerase chain reaction (RT–PCR).

Methods and Results: Housekeeping genes, 16S–23S rDNA and rpoS, as well as virulence genes, tdh1 and tdh2, were selected and detected by PCR in a pathogenic strain of V. parahaemolyticus (Vp4). Their expression was then studied by RT–PCR in V. parahaemolyticus Vp4 cultivated in rich medium at 37°C. The 16S–23S rDNA and rpoS, tdh1, tdh2 genes were transcripted at the mid-logarithmic, stationary and late stationary phases, corresponding to various physiological states. The expression of these genes was also studied by RT–PCR in a VBNC population of V. parahaemolyticus Vp4 in artificial seawater (ASW). The effect of temperature (washing of bacterial culture and microcosms) on the attaining VBNC bacteria was first considered. Washing of V. parahaemolyticus Vp4, collected at the mid-logarithmic phase, at 10 or 4°C before inoculation in ASW at 4°C allowed bacteria entered the VBNC state between 22 and 31 days. The 16S–23S rDNA and rpoS gene were expressed in the VBNC bacteria whereas no expression of the tdh1 and tdh2 genes was observed in the same populations.

Conclusion: The two selected housekeeping genes, 16S–23S rDNA and rpoS, proved to be good viability markers for V. parahaemolyticus Vp4 in culturable and VBNC states. These first data indicated that the pathogenic strain Vp4 would not maintain the expression of the virulence genes, tdh1 and tdh2, in VBNC state.

Significance and Impact of the Study: Use of RT–PCR for investigating the maintenance or not of viability and potential virulence in VBNC V. parahaemolyticus will facilitate further study to evaluate the potential risk presented by this pathogen in the environment.

Keywords: *Vibrio parahaemolyticus*; viable but non culturable state; RT-PCR; *tdh1*; *tdh2*; *16S-23S* rDNA; *rpoS*; environment.

INTRODUCTION

Vibrio parahaemolyticus is a gram negative marine bacterium known to be a significant human pathogen. Some strains are able to induce gastroenteritis after consumption of raw or undercooked contaminated seafood. Indeed, *V. parahaemolyticus* has been recognized as a major cause of foodborne gastroenteritis in Japan and linked to seafood consumption (Japanese National Institute of infectious Diseases 1999). In the United States, more than 700 cases of illness due to *V. parahaemolyticus* and associated to raw oyster consumption, were reported between 1997 and 1998 (DePaola *et al.* 2000). However, only a few outbreaks have been reported in Australia between 1990 and 1992 (Kraa 1995) and in France in 2001 (Haeghebaert *et al.* 2002). Most clinical strains of *V. parahaemolyticus* produce a major virulence factor, the thermostable direct hemolysin (TDH). These strains are hemolytic on Wagatsuma agar and designated as Kanagawa phenomenon positive (KP^+) (Miyamoto *et al*. 1969). Another virulence factor, the TDH-related hemolysin (TRH), is associated with KP^- strains (Oliver and Kaper 1997; Lesne and Fournier 1998). However, some strains associated with KP^- phenotype, can be tdh^+ (Nishibuchi and Kaper 1995). It was shown that culturability of *V. parahaemolyticus* changed with seasons (Chowdhury *et al.* 1990) and that this species entered into a viable but non culturable (VBNC) state in response to a low temperature of 4°C (Jiang and Chai 1996; Mizunoe *et al.* 2000). This physiological state was already described for many bacterial pathogens i.e., *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Shigella dysenteriae*, *V. cholerae*, *V. vulnificus* and other bacterial species (Oliver 2000). In the VBNC state, these bacteria keep their viability which is measured by classical methods such as the direct viable count (DVC) method (Kogure *et al.* 1979), the 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) and the 5-cyano-2,3-ditolyltetrazolium chloride (CTC) respiratory activity method (Zimmerman *et al.* 1979; Rodriguez *et al.* 1992) and the [^{3}H] leucine incorporation measurement (Lleo *et al.* 1998). The maintenance of potential virulence in the VBNC state is followed by using animal and human models (Oliver and Bockian 1995; Colwell *et al.* 1996) or immunological techniques such as ELISA (Rahman *et al.* 1996). During the last few decades, molecular methodologies like PCR and RT-PCR were developed. As opposed to DNA, mRNA was demonstrated to be a good viability marker due to its central role in bacterial physiology and its very short half-life (Sheridan *et al.* 1998). Reverse transcription-PCR (RT-PCR) was used as a viability test for VBNC populations of *Enterococcus faecalis* (Lleo *et al.* 2000). Recently, cytotoxin-hemolysin mRNA, encoding a hemolysin having cytotoxic activity against mammalian erythrocytes, was detected in VBNC populations of environmental and clinical *V. vulnificus* strains maintained in artificial seawater (ASW) (Fischer-Le Saux *et al.* 2002). This study indicated that RT-PCR was a good methodology to estimate the viability and the maintenance of potential virulence of bacteria population, simultaneously.

However, the maintenance of viability and potential virulence in the VBNC state has never been demonstrated for *V. parahaemolyticus* by RT-PCR. In this study, two housekeeping genes *16S-23S* rDNA and *rpoS* were chosen to detect the viability of *V. parahaemolyticus*. The *16S-23S* rDNA have intergenic spacers (IGS) which are not-translated sequences specific of *V. parahaemolyticus* (Maeda *et al.* 2000; Kong *et al.* 2002). The *rpoS* gene encodes a transcription factor named sigma S (σ^s) which regulates stress response mechanisms in *Escherichia coli* (Lowen and Hengge-Aronis 1994; Hengge-Aronis 2002). The *tdh1* and *tdh2* genes, encoding TDH (Iida and Yamamoto 1990; Nishibuchi and Kaper 1990), were chosen to detect the expressed virulence of *V. parahaemolyticus*.

The genetic expression of the *16S-23S* rDNA and the *rpoS*, *tdh1* and *tdh2* genes was estimated by RT-PCR in *V. parahaemolyticus* at various physiological states (viable

culturable and VBNC) in rich medium and under conditions resembling the natural marine environment.

MATERIALS AND METHODS

Bacterial strains and growth media

One strain of *V. parahaemolyticus* (Centre National de Référence des Vibrions et du Cholerae, CNRVC 990346, Vp4) was selected according to its human origin (clinical strain), the PCR amplification of the pR72H fragment and the presence of the virulence genes *tdh1* and *tdh2*. The pR72H fragment, non-coding region conserved in all strains of *V. parahaemolyticus* (Lee *et al.* 1995), allowed to confirm the biochemical identification of the bacteria (Hervio-Heath *et al.* 2002).
Bacteria were grown on Heart Infusion (HI) agar (0.5% or 2% NaCl) (Difco Laboratories, Detroit, Mich.) at 37°C and in HI broth (0.5% NaCl) at 37°C with shaking.
Strains of *V. vulnificus*, *V. cholerae*, *V. alginolyticus*, *Aeromonas sobria*, *Shewanella putrefaciens* and *Chromobacterium violaceum* were chosen to test the specificity of the primers used in this study.

Growth profiles in rich medium

(i) Inoculation of HI broth (0.5% NaCl). The selected *V. parahaemolyticus* strain was isolated on HI agar (0.5% NaCl). Then, a colony was inoculated in a 20 ml volume of HI broth overnight at 37°C. A 5 ml volume of this culture diluted ten times (10^{-1}) in HI broth was added to 495 ml of fresh HI broth and incubated at 37°C with shaking. The optical density (OD 610 nm) was recorded every 30 min and/or hour for eight hours. Culturable and total bacteria counts were determined at the same times. The mid-logarithmic phase as well as the stationary phase entry were determined for *V. parahaemolyticus* Vp4.
(ii) Total cell count. The total cell number was determined by direct counting after 4', 6 diamidino-2-phenylindole staining (DAPI; Sigma-Aldrich, Saint Quentin Fallavier, France) (Porter and Feig 1980). Samples were fixed with a 37% formalin solution (2% final concentration) and filtered through 0.22 µm pore size polycarbonate black filters (Dominique Dutscher S. A., Brumath, France). These filters were stained with 2 ml of DAPI solution (2.5µg ml^{-1}) for 20 min, washed with 3 ml of sterile water and then, mounted on a glass slide and observed using an epiflurescence microscope (Olympus, model BX60, SCOP group Olympus, Rungis, France) with a WU filter.
(iii) Culturable bacteria count. Colony-forming units (CFU) were enumerated by diluting 1 ml of the culture in buffered physiological saline (BPS). Decimal dilutions (0.1 ml) of the cell suspension were spread on HI (0.5% NaCl) agar in duplicate. Colonies were counted after incubation for 24 h at 37°C.
(iv) Viable bacteria count. This test is based on the integrity of the bacterial membrane (Kit Live/Dead® *Bac*Light™, L-7012, Molecular Probes, Leiden, The Netherlands). A solution was prepared with one volume of SYTO9 (3.34 mmol l^{-1}) and one volume of propidium iodide (20 mmol l^{-1}). One milliliter of the cell suspension was mixed with 3 µl of SYTO9/propidium iodide stain. After 15 min of incubation in the dark (room temperature), the bacterial suspension was filtered through a 0.22 µm pore size polycarbonate black filter. This filter was then mounted on a glass slide and observed using an Olympus epifluorescence microscope with a WB filter. All green cells were concidered viable while red cells were concidered dead.

Induction of VBNC population in artificial seawater

(i) Inoculation of artificial seawater (ASW). ASW flasks were prepared as described by Fischer-Le Saux *et al.* (2002). These flasks were inoculated with a mid-logarithmic phase bacterial culture (OD 610 nm of around 0.07) as follows: a 200 ml volume of *V. parahaemolyticus* Vp4 culture was centrifuged at 2500 g for 20 min at 4°C or 10°C, and then washed twice in sterile ASW at 4°C or 10°C. Washed bacteria were inoculated in a final two liter ASW volume in a five liter flask and then stored at 4°C. This protocol yielded a population of approximately 10^6 bacteria ml^{-1} in the inoculated ASW.
(ii) Monitoring entry into the VBNC state. The total and culturable bacteria counts were determined at day 0 (D0), D1, D2, D8, D15, D22, D31, and D42. The total bacteria count was performed as described above. However, CFU were enumerated by diluting 1 ml of the medium (ASW with *V. parahaemolyticus* Vp4) in sterile ASW, or after filtration (samples of 1 ml to 10 ml) through a 0.22 µm-pore-size nitrocellulose filter (Millipore, St Quentin en Yvelines, France). One hundred microlliters of these dilutions or the filters were spread or spread plated on HI agar (2% NaCl) in duplicate. Colonies were counted after incubation for 48 h at 37°C. The VBNC state was reached when one or less than one bacterium was enumerated in 20 ml of ASW (Fischer-Le Saux *et al.* 2002).

PCR detection of selected genes from *V. parahaemolyticus*

(i) DNA extraction. The DNA extraction was performed with the strains of *V. parahaemolyticus* and other bacteria as follows: 2 to 10 ml of bacterial culture in stationary phase were centrifuged at 2500 g for 3 min at room temperature. The bacterial pellet was solubilized in 1 ml of DNA Now solution (OZYME, Saint Quentin Yvelines, France) and vortexed vigorously for 1 min. Two hundred µl of chloroform were added and the sample was agitated by reversals for 20 sec and put on the ice for 5 min. After centrifugation (15 min at 2500 g at 4°C), one volume of isopropanol was added to one volume of the aqueous phase and the sample was precipitated for 45 min at -80°C. The DNA pellets were then washed twice with 1.5 ml of a 70% ethanol solution. The DNA pellet was vacuum-dried for 10 min and solubilized in 50 µl of sterile deionized water by heating (10 min at 60°C). This DNA solution was stored at -80°C until use.
(ii) Oligonucleotide primers. The primers used for the identification of *V. parahaemolyticus* (pR72H fragment) and the detection of various genes in *V. parahaemolyticus* and other bacteria as well as their expression in *V. parahaemolyticus*, are listed in table 1.

Table 1. PCR conditions used for each set of primers.

Target genes	Oligonucleotide sequences	Amplicon size (bp)	PCR conditions	Source
pR72H Fragment	VP33 (5'-TGC GAA TTC GAT AGG GTG TTA ACC-3') VP32 (5'-CGA ATC CTT GAA CAT ACG CAG C-3')	387 or 320	94°C 1 min 60°C 1 min 72°C 1 min	Lee *et al*. (1995)
16S-23S rDNA (IGS)	Vpara-F (5'-GCT GAC AAA ACA ACA ATT TAT TGT T-3') Vpara-R (5'-GGA GTT TCG AGT TGA TGA AC-3')	170	94°C 30 sec 55°C 30 sec 72°C 45 sec	Kong *et al*. (2002)
rpoS	VrposF (5'-GAC AAT GCG TCA GAG ACG-3') VrposR1 (5'-TCA CCA CGC AAT GCT CTG-3')	203	94°C 30 sec 56°C 30 sec 72°C 45 sec	This study
tdh1, *tdh2*	L-tdh (5'-GTA AAG GTG TCT GAC TTT TTG AC-3') R-tdh (5'-TGG AAT AGA ACC TTC ATC TTC ACC-3')	270	94°C 30 sec 58°C 30 sec 72°C 1 min	Hervio-Heath *et al*. (2002)
tdh1	VP21 (5'-TGG TTG ACA TCC TAC ATG ACT GTG-3') VP22 (5'-GGG GAT CCC TCA GTA CAA AGC CTT-3')	400	94°C 30 sec 55°C 30 sec 72°C 45 sec	Lee and Pan (1993)

tdh, thermostable direct hemolysin; IGS, intergenic spacer.

(iii) PCR conditions. PCR was conducted in a Peltier Thermal Cycler-200 (MJ Research, INC., Waltham, MA., USA). The different amplifications were optimized for a 40 µl mixture containing 1 X PCR buffer (10 mmol l^{-1} Tris-HCl, 1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl [pH 8.3]) (Roche Diagnostics, Meylan, France), 200 µmol l^{-1} of each deoxyribonucleotide triphosphate (dNTP), 1U of *Taq* DNA polymerase (Roche Diagnostics), 0.5 µmol l^{-1} of each primer and 200 ng of DNA extract or 4 µl of water (negative control) or 4 µl of purified DNA (positive control). The amplifications were performed as follows: an initial denaturation at 94°C for 5 min, 35 cycles of denaturation, annealing and extension (size of the amplicon and temperature conditions are described in table 1), and a final extension at 72°C for 7 min. A 15 µl aliquot of each amplification reaction mixture was electrophoresed through a 1.5% agarose gel Tris-EDTA buffer and visualized by ethidium bromide staining and UV illumination.

RT-PCR detection of selected genes in viable and culturable (VC) and VBNC populations of *V. parahaemolyticus*

(i) Total RNA extraction. The extraction was performed for culture samples or ASW samples inoculated with *V. parahaemolyticus* Vp4 using RNA Now solution (OZYME). For the culture, replicates of 2 and 10 ml (stationary and logarithmic phases, respectively) were centrifuged at 2500 g for 3 min at room temperature. For the ASW samples, various volumes (5 to 50 ml, duplicates) were filtered through a 0.22 µm-pore-size polycarbonate filter (47-mm diameter; Dominique Dutscher). The bacterial pellets or the filters were vortexed vigorously for 1 min in 1 ml and 2 ml of RNA Now solution, respectively. The filter was removed and the two suspensions were placed on ice for all the following steps. The extraction of total RNA was performed as specified by the manufacturer (RNA Now solution, OZYME) and was identical to DNA extraction with DNA Now solution. The RNA pellet obtained after the last 70% ethanol wash was dissolved in 30 or 50 µl of diethylpyrocarbonate-treated water (Sigma-Aldrich, Saint Quentin Fallavier, France) and

solubilized for 10 min at 60°C. The RNA extract was then stored at -80°C with 20 U of RNase inhibitor (Applied Biosystems, Courtaboeuf, France).
(ii) Elimination of contaminating DNA. In order to eliminate carryover DNA in the RNA extract, 25 µl of this RNA extract were treated for 1 h at 37°C with 150 U of DNase I, RNase-free (Roche Diagnostics) and 50 U of RNase inhibitor in a final volume of 100 µl. The DNase I was then inactivated by heating at 90°C for 5 min. The absence of DNA was subsequently tested by PCR for *16S-23S* rDNA (IGS) using 4 µl of DNase I-treated extract.
(iii) RT-PCR conditions. During the RT reaction, DNase I-treated RNA extract was incubated at 42°C for 15 min with 50 units of the murine leukemia virus reverse transcriptase (MulV RT; Applied Biosystems), heated at 95°C for 5 min and kept at 5°C until PCR. A 8 µl-volume of the DNase I-treated RNA was reverse transcribed in a mixture of 20 µl. The final concentrations were the following: 3.5 mmol l^{-1} $MgCl_2$, 1 X PCR buffer, 1 mmol l^{-1} each dNTP, 20 U of RNase inhibitor, 50 U of MulV RT and 1 µmol l^{-1} of antisense primer. After the RT, a PCR was performed using the conditions described previously (Table 1). The PCR mixture volume was adjusted to 50 µl and contained 40 µl of PCR mix and 10 µl or 6 µl (+ 4 µl of sterile DEPC-treated water) (adjustment in relation to the bacterial concentration in ASW sample) of the cDNA extract. The products of PCR were separeted on an agarose gel as previously described.

Lethal treatment

As previously described by Fischer-Le Saux *at al.* (2002), a lethal treatment (10 min at 100°C) was applied to verify that mRNA was a good viability marker. Briefly, presence of *rpoS* mRNA was tested by RT-PCR on RNA extractions performed in *V. parahaemolyticus* Vp4 VBNC population (D42), before and after a lethal treatment. Moreover, a 10 µl-volume of a RNA extract from *V. parahaemolyticus* Vp4 (stationary phase) was also treated for 10 min at 100°C and tested for *rpoS* mRNA presence.

Sequences alignments

The primers (VrposF and VrposR1), specific for the *rpoS* gene of *V. parahaemolyticus*, were selected from the alignment of *rpoS* gene sequences of *V. cholerae* (GenBank accession numbers AE004139, AF000945, D55632), *V. harveyi* (AF321124), *V. vulnificus* (AY163815) and *V. parahaemolyticus* (AF144608; primer VrposF, position 738-755 and primer VrposR1, position 940-923) using the CLUSTAL W (version 1.8) multiple-alignment program (Thompson *et al.* 1994).

RESULTS

Specificity of primers

The presence of the pR72H fragment unique to *V. parahaemolyticus*, and target genes, *16S-23S* rDNA, *rpoS*, *tdh*, and the specificity of selected primers (VrposF/VrposR1) were determined by PCR on DNA extracts from *V. parahaemolyticus* Vp4 and from other bacteria isolated from environmental samples. The presence of pR72H fragment was determined by the vizualization of a 320-bp amplicon, specific for *V. parahaemolyticus* (Hervio-Heath *et al.* 2002). The VP21/VP22 primers were used for the *tdh1* gene detection, and L/R-tdh primers for the detection of *tdh1* and/or *tdh2* genes. The amplification of a 400-bp fragment and a

270-bp fragment, respectively, in only one strain, Vp4, indicated the good specificity of these primers. The *rpoS* primers designed in this study for the detection of the *rpoS* gene (203-bp) were specific for *V. parahaemolyticus* (data not shown). Similarly, the *16S-23S* rDNA was only detected in *V. parahaemolyticus* (amplicon of 170-bp) indicating the good specificity of these primers for the IGS region.

Detection by RT-PCR of *16S-23S* rRNA, *rpoS* and *tdh* mRNA in *V. parahaemolyticus* cultived in rich medium

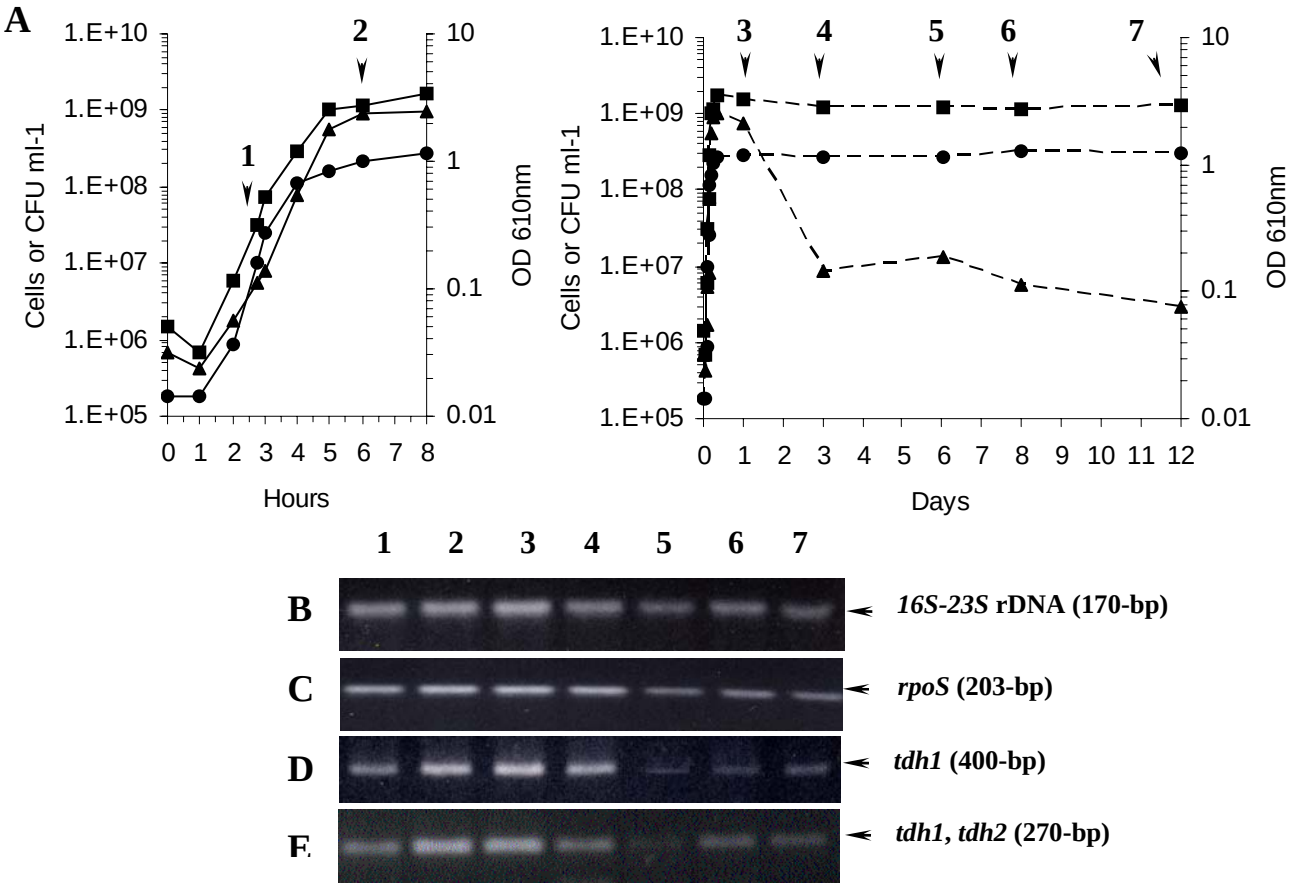
Growth curves were performed for *V. parahaemolyticus* Vp4. These curves indicated that the mid-logarithmic phase (1 to 5 x 10^7 CFU ml^{-1}; OD 610 nm between 0.1 and 0.2) was always observed 2 h 45 min after the beginning of the culture (T_{2h45}) and that the entry into the stationary phase was after 6 h (T_{6h}) (data not shown). These results were validated on other *V. parahaemolyticus* strains (environmental origin) and thus, allowed to have a reproducible protocol for studying precisely the genetic expression in *V. parahaemolyticus* at various physiological states.

The expression of the *16S-23S* rDNA, and of the *rpoS*, *tdh1* and *tdh2* genes was then followed for *V. parahaemolyticus* Vp4 cultivated for 12 days in rich medium (HI broth) at 37°C.

Three phases corresponding to three various physiological states were observed: a logarithmic phase (with bacteria in dividing bacillus shape) between T_{0h} and T_{6h}, a stationary phase (with bacteria in dividing bacillus and coccoidal shapes) between T_{6h} and day 1 (D1), and a late stationary phase (with bacteria in coccoidal shape) between D3 and D12 (Fig. 1A). At the mid-logarithmic phase, the total bacteria number was 3.12 x 10^7 bacteria ml^{-1} whereas it was constant and equivalent to 1.50 x 10^9 bacteria ml^{-1} during the stationary and late stationary phases. A decrease in culturability of two logarithmic units was recorded between D1 and D3 (Fig. 1A, points 3 and 4, respectively). Indeed, the culturable bacteria count varied from 7.65 x 10^8 CFU ml^{-1} (Fig. 1A, point 3) to 8.65 x 10^6 CFU ml^{-1} (Fig. 1A, point 4). A viability test (Kit Live/Dead® *Bac*Light™) was performed from D6 to D12 to investigate more accurately the stress effect in the late stationary phase,. At D6 and D8, the viable bacteria count was approximately one logarithmic unit higher than the culturable bacteria count. At D12, the viable bacteria count was 0.5 logarithmic unit higher than the culturable bacteria count (data not shown).

The expression of two housekeeping genes, *16S-23S* rDNA and *rpoS*, as well as that of the virulence genes, *tdh1* and *tdh2*, were followed by RT-PCR in bacterial populations collected during the logarithmic (T_{2h45}), stationary (T_{6h} and D1) and late stationary (D3, D6, D8 and D12) phases. During the logarithmic phase, the bacteria expressed the *16S-23S* rDNA and *rpoS* DNA (Fig. 1B, and 1C, respectively, lane 1). These two genes were still expressed during the stationary and late stationary phases (Fig. 1B and 1C, lanes 2 and 3, and 4 to 7, respectively). However, a slight decrease of the amplification signal was observed between D6 and D12 (Fig. 1B and 1C, lanes 5 to 7) whereas the total number of bacteria present in the different samples was the same. The *tdh1* gene was expressed in the logarithmic (Fig. 1D, lane 1), the stationary (Fig. 1D, lanes 2 and 3) and the late stationary (Fig. 1D, lanes 4 to 7) phases. Using L/R-tdh primers, an amplicon (270-bp) corresponding to the targeted sequences of *tdh1* and *tdh2* mRNA was observed in the same samples (Fig. 1E, lanes 1 to 7). However, a higher decrease of the amplification signal was observed for the *tdh* genes than for the two housekeeping genes between D6 and D12 (Fig. 1D and 1E, lanes 5 to 7).

Fig. 1 Culturability and transcription of *V. parahaemolyticus* Vp4 in HI (0.5% NaCl) broth at 37°C. (A) The graph to the left shows the logarithmic phase and the entry in stationary phase. The graph to the right shows the stationary and late stationary phases. The bacterial growth is followed at OD 610 nm (●). Total cells (■) counts and CFU (▲) are determined. Extractions of mRNA are performed on bacteria suspensions at different times (A, points 1 to 7 ⬇) and the expression of various genes: (B) *16S-23S* rDNA, (C) *rpoS*, (D) *tdh1* and (E) *tdh1*, *tdh2*, is followed by RT-PCR in Vp4 strain at different times: lane 1, T_{2h45}; lane 2, T_{6h}; lane 3, D1; lane 4, D3; lane 5, D6; lane 6, D8 and lane 7, D12.



Detection by RT-PCR of *16S-23S* rRNA, *rpoS* and *tdh* mRNA in a VBNC population of *V. parahaemolyticus* in artificial seawater

Figure 2 presents the loss of culturability of *V. parahaemolyticus* Vp4 population in artificial seawater (ASW) maintained at 4°C for 42 days (the bacteria were washed with ASW at 10°C or 4°C prior to inoculation in ASW flasks). The total and culturable cell counts were 2 x 10^7 cells ml^{-1} and 9.5 x 10^6 CFU ml^{-1}, respectively, in the HI Broth at the mid-logarithmic phase. The total and culturable counts were reduced to 2 x 10^6 cells ml^{-1} and 1.3 or 2.7 x 10^4 CFU ml^{-1} in the ASW flasks at D0 (for the washing at 4°C or 10°C, respectively) (Fig. 2). If we take the decimal dilution (at the time of ASW flask inoculation) into account, the loss of culturability is estimated to approximately 1.5 logarithmic units at D0. Sampling at D31 indicated that both populations were in the VBNC state, the detection limit of 0.05 CFU ml^{-1} being reached. This VBNC state was confirmed 11 days later (D42).

RNA extractions were performed simultaneously on ASW suspensions inoculated with *V. parahaemolyticus* Vp4 sampled from the two flasks (Vp4 cultures washed at 10°C and

4°C, respectively) at D0, D2, D15, D22, D31 and D42 (Fig. 2). An amplicon of 170-bp, indicating the presence of *16S-23S* rRNA, was observed after RT-PCR from D0 to D42 in the two ASW flasks (Fig. 3A, lanes 1 to 12). An amplicon of 203-bp was also detected in all samples for the *rpoS* gene (Fig. 3B, lanes 1 to 12). For these two genes, the expression was observed in VBNC bacterial population at D31 and D42 (Fig. 3A and 3B, lanes 5 and 11, and 6 and 12, respectively). No signal was detected for the *tdh1* mRNA in the Vp4 ASW samples collected at the same times between D0 and D42 (Fig. 3C, lanes 1 to 12) and this, despite the re-amplification performed on the RT-PCR products (data not shown). Similarly, no specific amplicon corresponding to the *tdh1* and *tdh2* mRNA was detected in the same samples when the L/R-tdh primers were used.

Fig. 2 Loss of culturability of *V. parahaemolyticus* Vp4 in ASW at 4°C. Total cell (■) and culturable (CFU) (▲) counts are performed in ASW flasks incubated at 4°C after a washing step at 10°C (full line) or 4°C (doted line). DL is the detection limit of the CFU (0.05 CFU ml^{-1}). The VBNC state is reached between 22 and 31 days (⊣ ⊢).

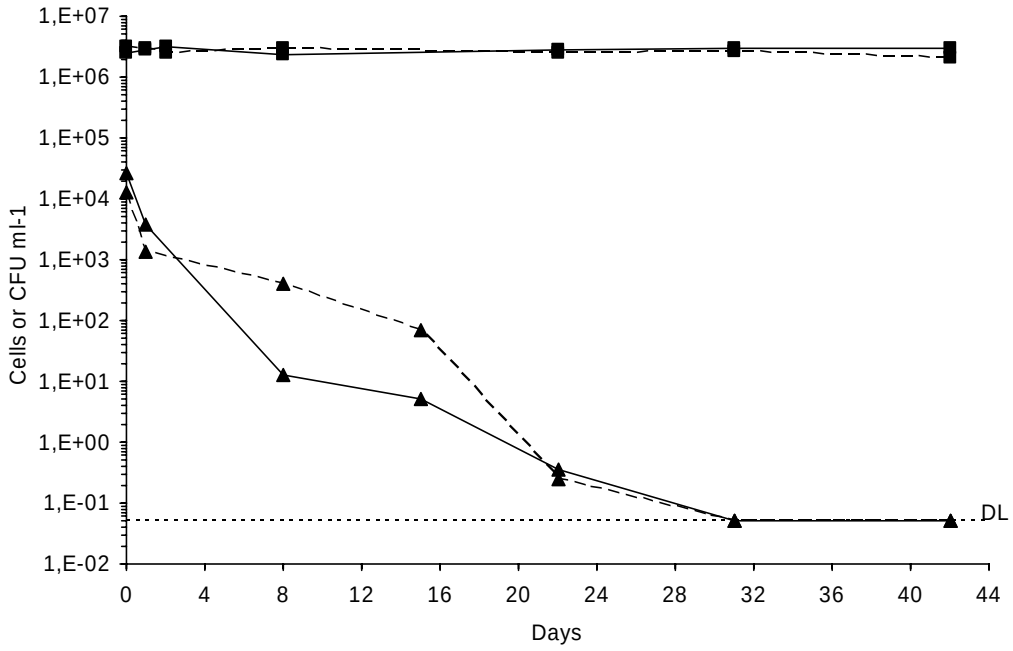
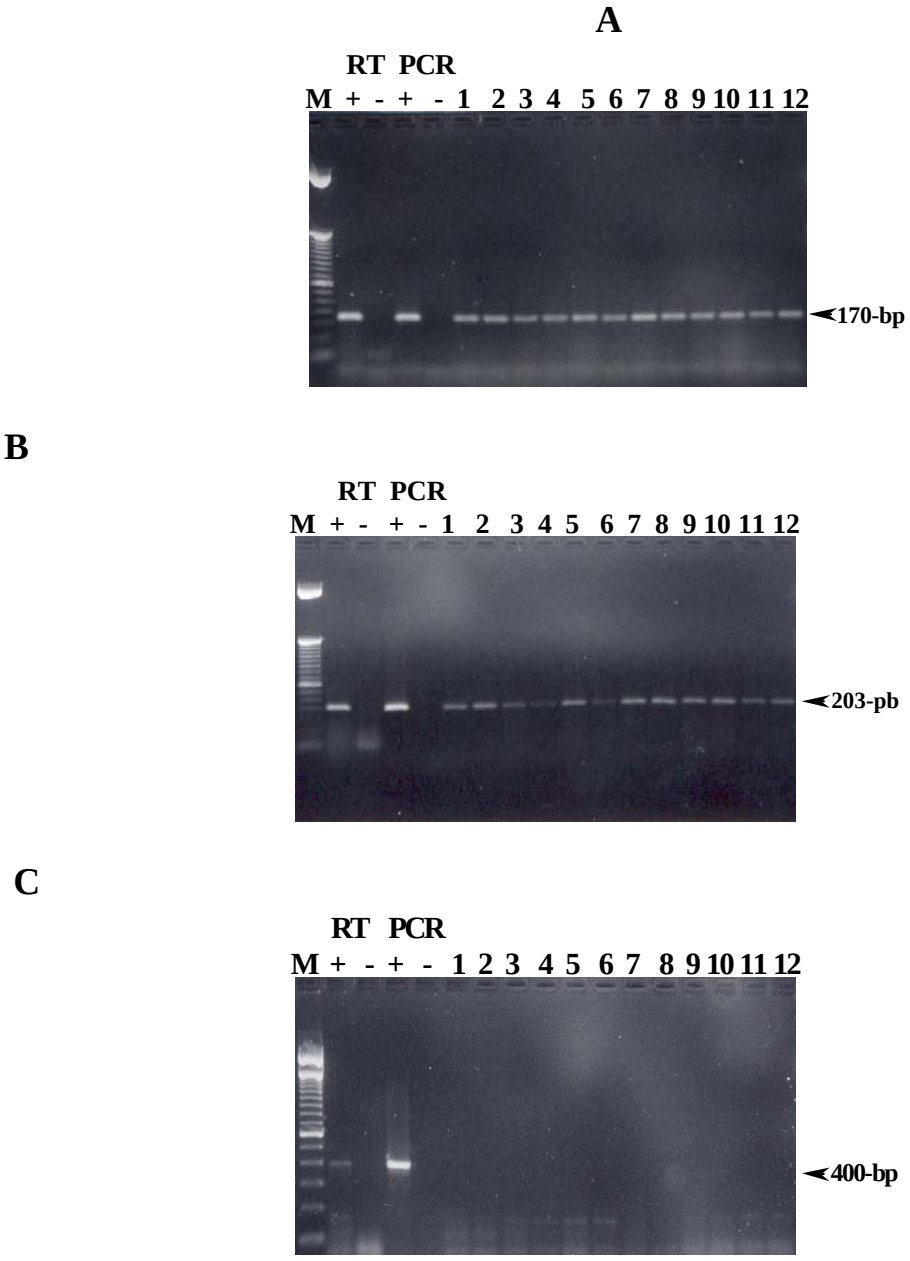


Fig. 3 Detection of *16S-23S* rRNA (A), *rpoS* mRNA (B), and *tdh1* mRNA (C) by RT-PCR on *V. parahaemolyticus* Vp4 in ASW incubated at 4°C after washing the culture at 10°C (Lanes 1 to 6) or 4°C (lanes 7 to 12). The extractions were performed at D0 (lanes 1 and 7), D2 (lanes 2 and 8), D15 (lanes 3 and 9), D22 (lanes 4 and 10), D31 (lanes 5 and 11) and D42 (lanes 6 and 12). The bacteria were in a VBNC state at D31 and D42. Positive controls (lanes +) of RT and PCR contained RNA and DNA of *V. parahaemolyticus* Vp4, respectively, whereas negative controls (lanes -) contained deionized water. M is a 50-bp (A and B) or a 100-bp DNA ladder size marker (C) (Invitrogen, Cergy Pontoise, France).

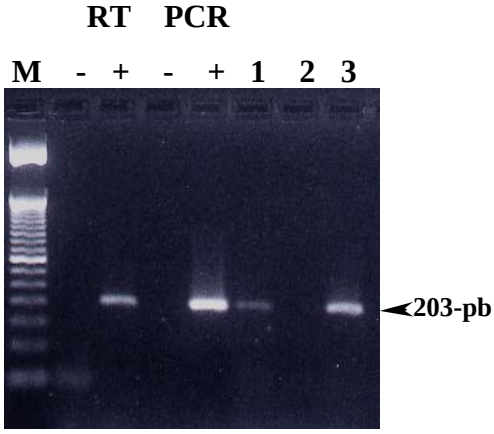


Lethal treatment

To verify that the mRNA presence was linked to the bacteria viability, RNA extractions were performed in a VBNC population of *V. parahaemolyticus* Vp4 from ASW

flask (at D42) before and after a lethal treatment. An amplicon of 203-bp was detected for the *rpoS* mRNA in the sample from VBNC with no lethal treatment (Fig. 4, lane 1). However, no signal was observed when the VBNC population was boiled at 100°C for 10 min (Fig. 4, lane 2). An amplicon of 203-bp was obtained on RNA extract subjected to the same lethal treatment (Fig. 4, lane 3).

Fig. 4 Detection of *rpoS* mRNA by RT-PCR in a VBNC population of *V. parahaemolyticus* Vp4 treated or not by heating. RT-PCR reactions are performed with the primers VrposF/VrposR1 on RNA sample of *V. parahaemolyticus* Vp4 in VBNC state (D42) (lane 1); on the same VBNC population killed by heat treatment at 100°C for 10 min (lane 2); and on RNA extract from *V. parahaemolyticus* Vp4 in stationary phase, treated at 100°C for 10 min (lane 3). Positive controls (lanes +) of RT and PCR contained RNA and DNA of *V. parahaemolyticus* (IF Vp18), respectively, whereas negative controls (lanes -) contained deionized water. M is a 50-bp DNA ladder size marker (Invitrogen).



DISCUSSION

In unfavorable conditions (low temperature, carbon or nitrogen starvation, etc), *Vibrio* can not be cultivated on the classical media (Huq and Colwell 1995). This physiological state called viable but non culturable (VBNC), was described for the first time with *Salmonella enteritidis* by Roszack *et al.* (1984). Some strains of *V. parahaemolyticus* being pathogens, it was interesting, in term of public health, to estimate their potential virulence in this VBNC state in a marine environment.

As opposed to numerous viability tests which are often subjected to controversy (Villarino *et al.* 2000), mRNA detection by RT-PCR is the most promising technique for investigating the maintenance of viability for bacteria in various physiological states i. e., viable culturable (VC) (Klein and Kuneja 1997; Sheridan *et al.* 1998) and VBNC (Lleo *et al.* 2000; Fischer-Le Saux *et al.* 2002). Although previous experiments indicated that time of mRNA (*groEL*, *rpoH*, *tufA*, *fliC*, *stx1* and *rfbE*) disappearance varied with lethal treatment and post-incubation conditions (Sheridan *et al*. 1998; Sheridan *et al.* 1999; Yaron and Matthews 2002), it was suggested that a positive amplification must be attributed to viable bacteria and cells dead recently. We first studied the expression of various genes in populations of a pathogenic strain *V. parahaemolyticus* Vp4 (*tdh1*$^+$, *tdh2*$^+$) cultivated in rich medium. Various growth curves were performed and three phases; a logarithmic, a stationary and a late stationary phase each being attributed to a different physiological state. During the late stationary phase, more viable bacteria were observed than culturable bacteria, suggesting that a part of viable bacteria was nonculturable. We then evaluated the genetic expression of two housekeeping

genes (*16S-23S* rDNA and *rpoS*) and of two virulence genes (*tdh1* and *tdh2*) in the *V. parahaemolyticus* Vp4 in these various physiological states. A control PCR was performed on total RNA extract pre-treated with RNase-free DNase I before each RT-PCR to ensure the specificity of the mRNA detection. The two housekeeping genes were expressed during the three phases. The *rpoS* mRNA were notably detected by RT-PCR in carbon starvation (and high bacterial density) i. e., during the stationary and late stationary phases, confirming that the regulation of this gene was post-transcriptional in these conditions like it was observed in previous studies on *E. coli* (Lange and Hengge-Aronis 1994; Hengge-Aronis 2002). In parallel, we observed that the virulence genes (*tdh1* and *tdh2*) were expressed in the same bacterial populations. A high decrease in the amplification signals was noted for these two genes during the late stationary phase. This could be explained by a partial or total repression of the *tdh* genes transcription resulting from the possible transition of a part of this bacteria population towards a VBNC state. In a second step, it was of interest to evaluate the expression of these genes in VBNC population of *V. parahaemolyticus* maintained in artificial seawater (ASW).

Following initial experiments using rich medium, we attempted to obtain the VBNC state in *V. parahaemolyticus* Vp4. Previous studies have shown that a decrease of water temperature was the main factor used to obtain *V. parahaemolyticus* in VBNC state (Jiang and Chai 1996; Mizunoe *et al.* 2000; Wong and Wang 2004). Our initial attempts performed with bacteria taken in the mid-logarithmic phase and washed in ASW at room temperature, did not allow to observe a marked decrease of the culturability of *V. parahaemolyticus* Vp4 maintained in ASW flasks at 4°C over 21 days (data not shown). However, when bacteria were washed with ASW at 10°C or 4°C prior to inoculation in ASW, a loss of culturability was observed in ASW flasks at 4°C. Thus, the bacteria washed in carbon starvation conditions (ASW) at room temperature could induce stress responses and these may repress the VBNC mechanism displayed during the 4°C incubation in the ASW flasks. Sampling at D31 indicated that both *V. parahaemolyticus* Vp4 populations were in the VBNC state. This one was probably reached before, the previous samples being D22 in the two flasks (0.35 CFU ml^{-1} and 0.25 CFU ml^{-1}, the washing at 10°C and 4°C, respectively). This is a lot sooner than for Jiang and Chai (1996) who obtained VBNC population of *V. parahaemolyticus* in 50 to 80 days but comparable to the results of Wong and Wang (2004) who obtained *V. parahaemolyticus* in the VBNC state within 28 to 35 days. However, in this latter study the population of *V. parahaemolyticus* VBNC was obtained using a minimum medium with 0.5% of NaCl. This NaCl concentration being weak for halophilic bacteria living in seawater, the VBNC state obtained in our study would thus, be closer to a VBNC state of *V. parahaemolyticus* in a marine environment. Variations could be explained by differences in the washing temperature, the strains or the media. Our results are more in agreement with those obtained by Baffone *et al*. (2003), Fisher-Le Saux *et al.* (2002) and Lleo *et al.* (2000) for *V. parahaemolyticus*, *V. vulnificus* and *Enterococcus faecalis*, respectively, using similar conditions. When a low temperature wash in carbon starvation conditions was applied to *V. parahaemolyticus* Vp4, a decrease of the culturability was observed in ASW flasks at 4°C. Washing of bacteria with a carbon deprived solution at 10°C or 4°C prior to inoculation in ASW could prevent adaptation of the bacteria to this carbon starvation. Indeed, those would be then stressed in ASW flasks at 4°C and lose their culturability. Our results are in agreement with those described in a previous study performed on *V. vulnificus* in ASW microcosms (Oliver *et al.* 1991).

Recently, Lleo *et al.* (2000) reported the expression of a gene involved in peptidoglycan synthesis in VBNC forms of *Ent. faecalis*. Here, we demonstrate that *V. parahaemolyticus* can also maintain gene expression in VBNC state. Indeed, the two housekeeping genes, *16S-23S* rDNA and *rpoS*, were expressed in *V. parahaemolyticus* Vp4 in the VBNC state. The

important role of the *16S-23S* rRNA in the mechanisms of translation could explain its expression in the VBNC bacteria. As for the RpoS factor, it has been shown to have a protector effect on the culturability of *E. coli* (Gourmelon *et al*. 1997) and S*almonella typhimurium* inoculated in oligotrophic medium (Munro *et al.* 1995) as well as on that of *V. cholerae* (Yildiz and Schoolnik 1998). Total RNA extractions and detection of *rpoS* mRNA by RT-PCR indicated that the loss of viability was well correlated with the loss of the RT-PCR signal in the heat-killed bacteria. Thus, the validity of RT-PCR as a tool to confirm the viability, already demonstrated in previous studies (Klein and Kuneja 1997; Sheridan *et al.* 1998; Lleo *et al.* 2000), was confirmed in this study for *V. parahaemolyticus* Vp4 VBNC populations.

However, no expression of the *tdh1* and *tdh2* genes was obtained for the same VBNC populations of *V. parahaemolyticus* Vp4. Expression of these genes in VBNC bacteria could be totally repressed or very weak and thus, not detectable by RT-PCR. These results confirm those obtained for *V. parahaemolyticus* Vp4 in the late stationary phase but are opposed to those obtained for *V. vulnificus* which express the *vvhA* gene coding for a virulence factor (hemolysin) in a VBNC state for three months (Fischer-Le Saux *et al.* 2002). Because the basal-level expression of *tdh2* gene is 21- to 26-fold higher than the one of *tdh1* gene (Lin *et al.* 1993) and because the *thd2* gene is at the origin of more 90% of the total TDH protein (Nishibuchi and Kaper 1995), it would be of interest to design specific new primers for the *tdh2* gene only. Futhermore, only the population of mRNA *tdh2* would be targeted and would thus, increase the sensitivity of the RT-PCR.

In conclusion, specific primers for the *rpoS* gene of *V. parahaemolyticus* have been determined. It is the first time that the expression of various genes was evaluated by RT-PCR in a pathogenic strain of *V. parahaemolyticus*. Because of the maintenance of their expression in stressed bacteria (late stationary phase and VBNC state), *16S-23S* rRNA and *rpoS* mRNA appeared as good viability markers for this *V. parahaemolyticus* strain (Vp4). A decreased transcription of the virulence genes (*tdh1* and *tdh2*) was reported in viable and culturable populations in stressed conditions. This is confirmed when the bacteria are in the VBNC state in artificial seawater. The observation that the *tdh1* and *tdh2* mRNA are not detected in VBNC population of *V. parahaemolyticus* is an interesting result in term of public health. However, various other genes, potentially involved in the *V. parahaemolyticus* pathogenicity, have to be tested. Moreover, these results have to be confirmed for other pathogenic strains of *V. parahaemolyticus*.

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