Quantitative reverse transcription polymerase chain reaction analysis of *Vibrio cholerae* cells entering the viable but non-culturable state and starvation in response to cold shock

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Summary

We performed a comparative analysis of the *Vibrio cholerae* strain El Tor 3083 entering the viable but non-culturable (VBNC) state and starvation after incubation in artificial seawater (ASW) at 4 and 15°C respectively. To this end, we determined bacterial culturability and membrane integrity, as well as the cellular levels of 16S rRNA and mRNA for the *tuf*, *rpoS* and *relA* genes, which were assessed by real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR). Bacterial cells entering the VBNC state showed a 154, 5.1×10^3, 24- and 23-fold reduction in the number of copies of 16S rRNA and mRNA for *tuf*, *rpoS* and *relA*, in comparison to exponentially growing cells. The differences were less striking between cells in the VBNC and starvation states. The mRNA for *relA* was selectively increased in VBNC cells (3.2-folds), whereas a 3.9-fold reduction was observed for 16S rRNA. The obtained results confirmed that key activities of the cellular metabolism (i.e. *tuf* representing protein synthesis, and *relA* or *rpoS* stress response) were still detected in bacteria entering the VBNC state and starvation. These data suggest that the new Q-RT-PCR methodology, based on the selected RNA targets, could be successfully exploited for the identification (rRNA) of *V. cholerae* and assessment of its metabolic activity (*tuf*, *rpoS*, *relA* mRNA) in environmental samples.

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Introduction

Vibrio cholerae is one of the most important waterborne pathogens (Kaper *et al*., 1995; Colwell, 1996; Faruque *et al*., 2003a; Jabeen and Hasan, 2003). In fact, cholera epidemic outbreaks have killed millions of people and continue to be a major public health concern worldwide (Faruque *et al*., 2003b). *Vibrio cholerae* is part of the normal microflora in estuarine and marine environments (Kaper *et al*., 1995; Colwell, 1996; Gil *et al*., 2004). These bacteria can also replicate within mussels, amoebae and snails, which provide environmental reservoirs for infection (Peterson, 2002; Reidl and Klose, 2002). Additional studies have demonstrated that *Vibrio* spp. are able to survive in water for quite a long time, and that even bacteria which have lost their culturability can still cause infections (Oliver, 1995).

The capacity of *V. cholerae* for long-term survival in water in either a viable but non-culturable (VBNC) state (Ravel *et al*., 1995; Pruzzo *et al*., 2003) or under starvation conditions (Wai *et al*., 1999; Pruzzo *et al*., 2003) renders the control of epidemic outbreaks difficult. This capacity seems to be related to the influence of many environmental factors, among them, temperature seems to play a crucial role (Pruzzo *et al*., 2003). The inoculation of *V. cholerae* in artificial seawater (ASW) and subsequent incubation at two different temperatures (5 or 15°C) results in their entrance into two different physiological states: (i) the VBNC state [i.e. < 0.1 colony-forming units per millilitre (cfu ml^{-1})] at 5°C and (ii) starvation (i.e. culturability is maintained) at 15°C (Pruzzo *et al*., 2003).

Bacteria that have reached the VBNC state are unable to grow in conventional media, but still maintain their viability (Lowder *et al*., 2000; Pruzzo *et al*., 2003). According to many studies, pathogenic bacteria in the VBNC state retain their virulence (Oliver and Bockian, 1995; Lleo *et al*., 2000; Baffone *et al*., 2003). In fact, previous work has demonstrated that VBNC cells of *V. cholerae* retain their pathogenic potential for a prolonged time (Colwell, 1996; Chaiyanan *et al*., 2001; Pruzzo *et al*., 2003). Thus, culture-independent detection and activity assessment methodology for environmental samples are much in

demand for a better understanding of the ecology of this waterborne pathogen. The protein profiles of *V. cholerae* cells entering the VBNC state in response to cold shock have been previously analysed (Hood *et al*., 1986; Carrol *et al*., 2001). However, proteins are more stable and have a longer half-life inside the cell than bacterial RNAs. Thus, RNAs are more suitable as cell viability markers for the study of the VBNC state than proteins (Sheridan *et al*., 1998; Lleo *et al*., 2000; Fischer-Le Saux *et al*., 2002). This fact would imply that as long as VBNC bacteria are alive, they would produce some mRNA molecules, especially those corresponding to stress response genes (e.g. *rpoS*, *relA*). In addition, other RNA present in high abundances in bacterial cells, such a ribosomal RNA or mRNA of the elongation factor TU, could function as indicator of cellular activity or marker for the presence of specific bacterial cells.

The main aim of our study was to develop a set of real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) methods applicable to the environmental study of *V. cholerae* in VNBC populations and under starvation conditions. Therefore, we selected 16S rRNA, and mRNA of the *tufA/B*, *rpoS* and *relA* genes as molecular targets to explore the utility of the total RNA fraction for culture-independent detection of active bacterial cells. The 16S rRNA was selected as representative of ‘stable’ RNA (Deutscher, 2003). The mRNA coding for the *tufA/tufB, relA* and *rpoS* genes were chosen because they are involved in key metabolic processes, such as protein translation for *tuf* (Manganelli *et al*., 2001; Schnell *et al*., 2003), and stress response for *relA* and *rpoS* (Flardh *et al*., 1994; Yildiz and Schoolnik, 1998). Because the lowering of temperature can lead to two different responses in *V. cholerae*, we performed a comparative quantitative evaluation of 16S rRNA and the selected mRNA (i.e. for the *rpoS*, *relA* and *tuf genes*) in bacterial cells entering the VBNC and starvation states, resulting from cold shock as defined above (Pruzzo *et al*., 2003). During this cold shock experiments, total RNA was isolated, and the cellular levels of the four targeted RNA were determined by a two-step real-time Q-RT-PCR. Studies were performed in parallel, to assess bacterial viability and culturability of the *V. cholerae* cells. To obtain an accurate determination of the RNA targets present in viable cells, we used RNA standards obtained by *in vitro* transcription, according to our recently established method (Fey *et al*., 2004). Our data confirmed that key components of the metabolism are maintained functional in bacteria entering the VBNC state and starvation, and that the newly developed Q-RT-PCR methods can be successfully exploited for the identification of *V. cholerae* and the assessment of its metabolic activity in environmental samples.

Results and discussion

Assessment of V. cholerae *survival in ASW*

To study the process of entering the VBNC state and starvation, we performed a comparative kinetic analysis of bacterial cells that were incubated in ASW using three different methods: (i) DAPI staining was used to determine the total number of stained particles (i.e. bacterial cells); (ii) determination of the number of cfu ml^{-1} by agar plating was done to establish the number of culturable cells able to grow in conventional culture media (TSA), and (iii) the BD™ cell viability kit was employed to determine by flow cytometry the number of viable cells (i.e. bacteria with intact membranes), independently of their growth potential. When exponentially growing cells of the *V. cholerae* strain 3083 were incubated in ASW at 4°C and 15°C, two different responses were observed (Fig. 1A). After 24 h a rapid loss of culturability could be observed at both incubation temperatures (i.e. 1 and 2 logs at 4 and 15°C respectively). This loss of culturability correlated with a

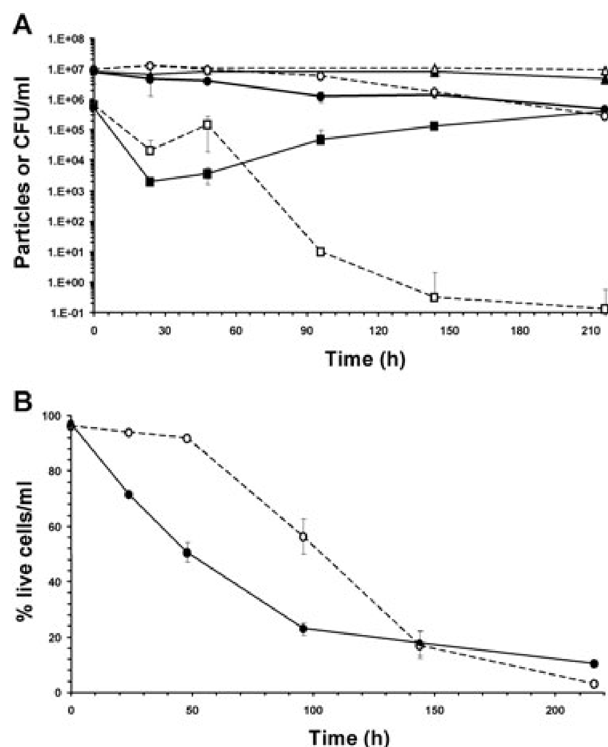

Fig. 1. Survival of *V. cholerae* cells entering the VBNC and starvation states.
A. The total number of particles per millilitre was determined in bacteria incubated in ASW by DAPI staining (▲), number of viable cells was determined by live/death stain and cytofluorimetric analysis (●), and culturability was assessed by agar plating (cfu ml^{-1}) (■).
B. Bacterial viability, as determined by assessing membrane integrity by flow cytometry, is expressed as percentage in respect to the initial values, which were arbitrarily considered as 100%. Empty and filled bullets represent the data generated using samples from bacteria incubated at 4 and 15°C respectively.

Table 1. Primers used to generate DNA and RNA standards and PCR primers for Q-RT-PCR.

Target gene	Primer name	Sequence (5′→3′)[a]	Position on gene[b]	Product size (bp)	Accession no.[c]
Generation of standards					
16S rRNA	Vc 16S f-T7	TAATACGACTCACTATAGGGCC TAGCTGGTCTGAGA	278–293	771	VCr001
	Vc 16S r-T7	ATGCAGCACCTGTCTCAGA	1031–1049		
*tuf*A and B	Vc *tuf* f-T7	TAATACGACTCACTATAGGGTC ATGAACAAGTGTGACATGGT	401–422	693	VC0321; VC0362
	Vc *tuf* r-T7	GCAATCAGGTCTACAACCATCT	1073–1094		
rpoS	Vc *rpoS* f-T7	TAATACGACTCACTATAGGGTG AGTGTCAGCAATAC	2–17	643	VC0534
	Vc *rpoS* r-T7	CGCAGCATCTTAGTGACATC	625–644		
relA	Vc *relA* f-T7	TAATACGACTCACTATAGGGTT GGTCAGCTCAAATGGGAA	569–588	686	VC2451
	Vc *relA* r-T7	GCCCATAGGTAAGTCGACCA	1235–1254		
PCR primers					
16S rRNA	Vspp-16SF-156	CGTAAAGCGCATGCAGGTG	562–580	162	VCr001
	Vspp-16SR-157	CTTCGCCACCGGTATTCCTT	704–723		
*tuf*A *tuf*B	Vc *tuf* f-st	ACTTATATTCCAGAGCCAGAGC	592–613	180	VC0321
	Vc *tuf* r-st	TGTACAGGTCGTTTTTACTGTC	750–771		
rpoS	Vc RpoS f 151	GCTTCTGCGAAAAGTCTTG	151–169	199	VC0534
	Vc RpoS R331	GTGCTAATCCTCGGTTGCT	331–349		
relA	Vc *relA* f st	ATGCAGAAAAAGAGCCTCGC	784–803	163	VC2451
	Vc *relA* r st	TCGGTTTTGGGTTTGCTACA	927–946		

a. Sequences corresponding to the T7 promoter are underlined.
b. Positions of genes are given according to the accession numbers.
c. Accession number at the NCBI (http://www.ncbi.nih.gov/).

fast reduction in bacterial viability (26%) with respect to the initial time for the cells incubated at 15°C (Fig. 1A and B). On the other hand, when bacteria were incubated at 4°C for 24 h, only 4% of the cells have lost their membrane integrity. However, a continuous reduction in bacterial viability was observed for cells incubated at both temperatures during the course of the experiment, which was faster for bacteria incubated at 15°C (Fig. 1B). Despite the decreased membrane integrity observed at 15°C, an increase in bacterial culturability (cfu ml^{-1}) was observed during the following days. At the end of the 15°C experiment, the cfu ml^{-1} were in agreement with data obtained by the cytofluorimetric viability test (i.e. approximately 10^6 viable cells ml^{-1}). At day 9, the cfu ml^{-1} of the cells incubated at 4°C were < 0.1 ml^{-1} and bacteria were considered to be in the VBNC state (Fig. 1A). Approximately 11% and 3% of the bacterial cells retained their membrane integrity at 15°C and 4°C respectively (Fig. 1B). These results demonstrate that incubation at the two different temperatures resulted in the intended effects after 9 days: (i) 4°C incubation in VNBC cells, and (ii) 15°C incubation in starvation conditions.

Evaluation of the Q-RT-PCR methods

The RNA standards, generated by PCR from genomic DNA (see *Experimental procedures*) were evaluated by Q-RT-PCR using the PCR primers specific for the *rpoS*, *relA*, *tuf* and 16S rRNA genes of *V. cholerae* (Table 1). In all cases we obtained linear calibration curves, which had a correlation coefficient (R^2) of ≥ 0.996, with linear ranges of ≥ 8 orders of magnitude for *rpoS*, ≥ 6 orders of magnitude for *relA*, ≥ 7 orders of magnitude for *tuf*, and ≥ 6 orders of magnitude for 16S rRNA (Fig. 2). This corresponds to detection limits of 8 mRNA copies for *rpoS*, 40 mRNA copies for *relA*, 50 mRNA copies for *tuf* and up to 270 rRNA copies for 16S rRNA (Table 2). The efficiency of the RT-PCR ranged from 0.79 to 0.94 (Table 2). Melting curve analyses, performed for each Q-RT-PCR, showed a clear melting peak (Table 2) and lack of unspecific products.

Quantification of the levels of 16S rRNA, and mRNAs for the rpoS*,* relA *and* tuf *genes in* V. cholerae *cells*

The presence of specific mRNA and 16S rRNA was determined in cells of *V. cholerae* incubated at 4°C and at 15°C

Table 2. Main properties of the primer sets used for Q-RT-PCR analysis.

Target	Reaction efficiency[a]	Melting temperature (°C)	Detection limit[b]
16S rRNA	0.94 ± 0.07	83.4	270
tuf mRNA	0.79 ± 0.01	84.6	50
rpoS mRNA	0.82 ± 0.07	84.3	8
relA mRNA	0.85 ± 0.02	83.6	40

a. Reaction efficiencies are mean values of three or more determinations ± standard deviations.
b. Number of copies.

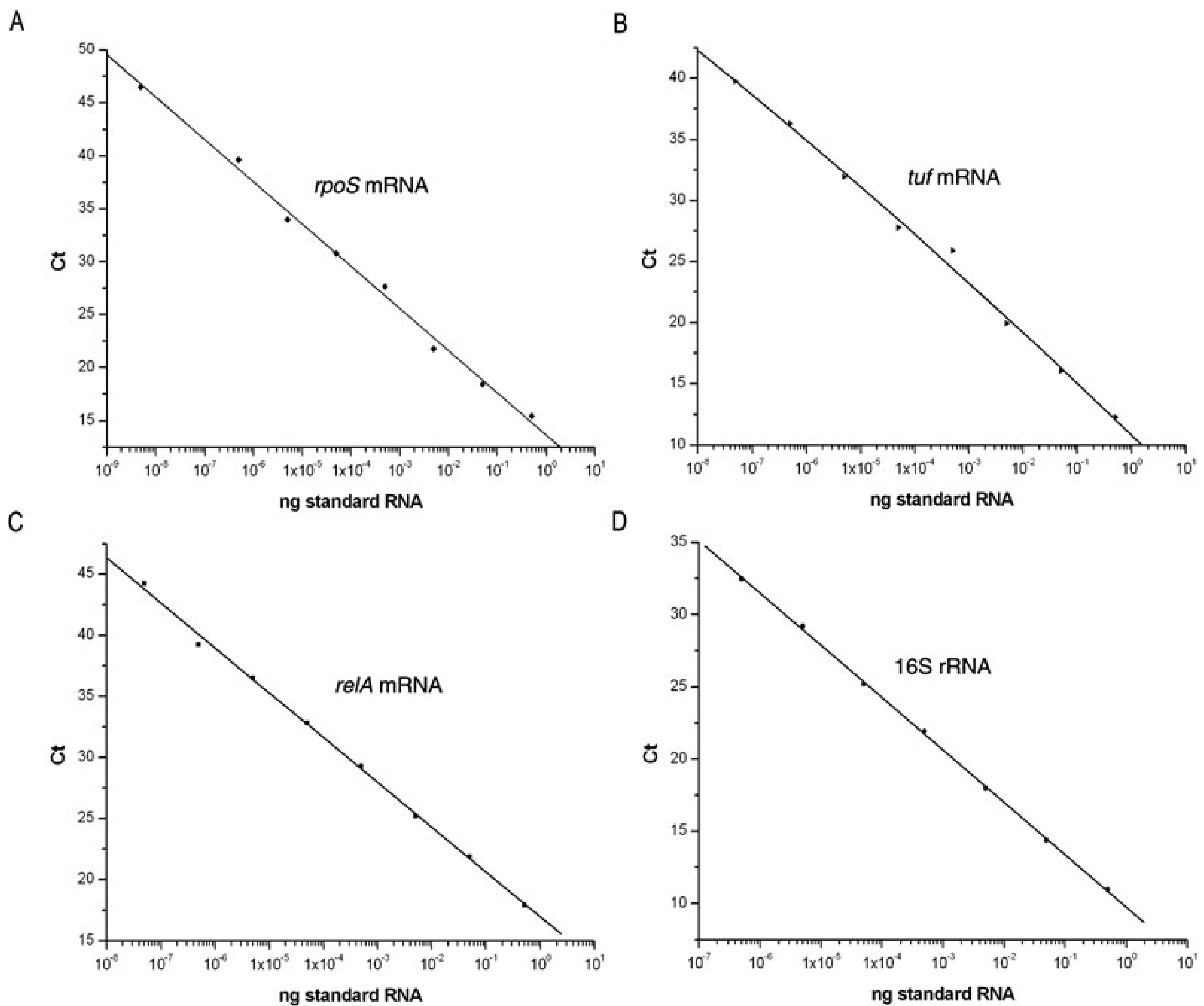

Fig. 2. Calibration curves generated using the RNA standards for *rpoS* (A), *tuf* (B), *relA* (C) and 16S rRNA (D). The target nucleic acid concentrations are plotted against the C_t values. The C_t is the cycle number at which the fluorescence in the sample increases above a defined threshold fluorescence, which is calculated by the software.

during 9 days until they reach the VNBC state and starvation (Fig. 3A–D). The number of 16S rRNA molecules ranged from about 700–4000 copies per live cell with no significant differences between the two temperatures (Fig. 3A). The number of *tuf* mRNA molecules per cell decreased rapidly within 2 days at both temperatures with higher values in the samples that were incubated at 4°C (Fig. 3B). The copy numbers of *rpoS* mRNA decreased also in parallel during the first 2 days, with higher values in the 4°C samples (Fig. 3C). A slight recovery of *rpoS* mRNA was observed after 6 days with no significant differences among the different temperatures. The copy number of *relA* mRNA remained low for 6 days with no significant differences between incubation temperatures (Fig. 3D). After 9 days the VNBC cells showed a threefold increase in comparison to the starved cells (Fig. 3D, Table 3).

The kinetic results given in Fig. 3 were compared with those from *V. cholerae* cells growing exponentially (Table 3). For comparative reasons, the end points after 9 days for the VNBC state and the starvation conditions were used. The number of RNA copies per live cell was 6.5, 272, 7.6 and 1.1×10^5 for *rpoS*, *tuf*, *relA* and 16S rRNA in exponential growing cells (Table 3). In contrast, bacteria entering the VBNC state showed 154, 5.1×10^3, 24- and 23-fold reduction in the number of copies of 16S rRNA and mRNA for *tuf*, *rpoS* and *relA*, with respect to exponentially growing cells. Thus, the metabolic activities related to protein synthesis seem extremely affected in VBNC cells (i.e. 16S rRNA and *tuf*). Regardless the ele-

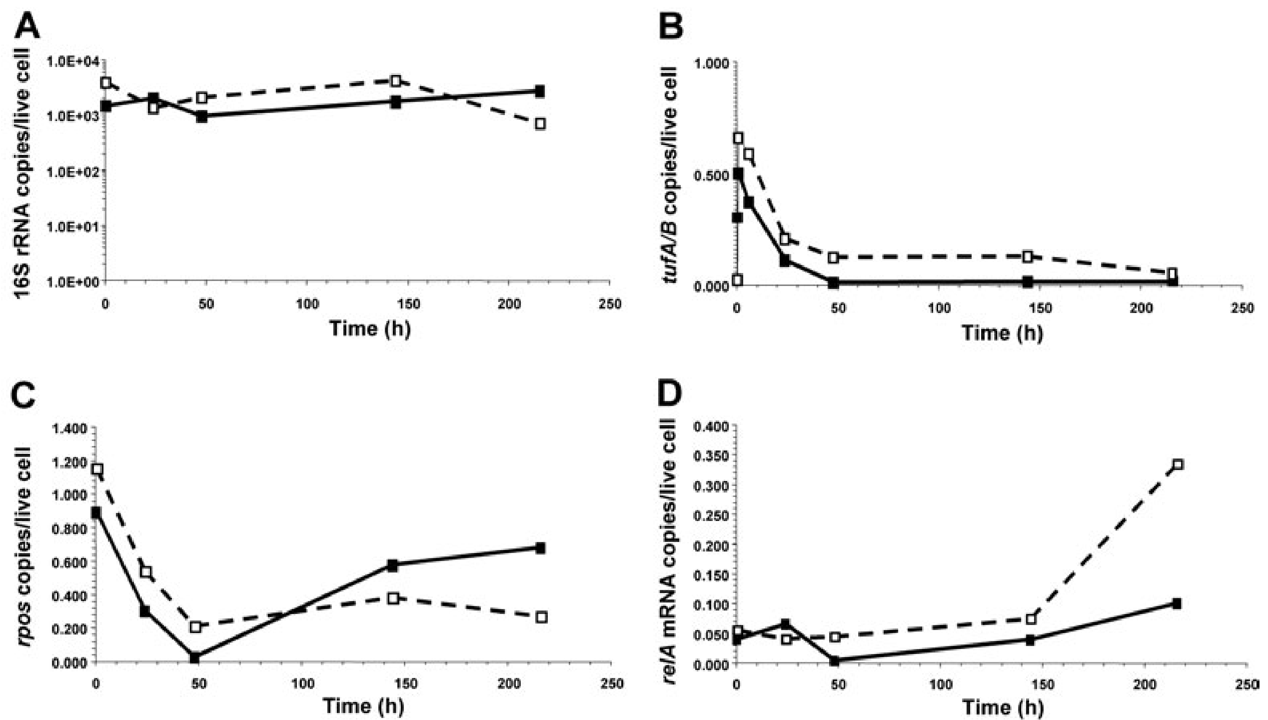

Fig. 3. Assessment of abundances of specific RNA by Q-RT-PCR analysis of *V. cholerae* cells entering the VBNC state and starvation. (A) 16S rRNA, (B) *tuf* mRNA, (C) *rpoS* mRNA and (D) *relA* mRNA. Live cells were measured as total number of particles retaining membrane integrity, according to the live/death staining. Empty and filled symbols represent the data generated using samples from bacteria incubated at 4 and 15°C respectively.

vated reduction observed in the expression levels for all the RNAs tested, they were still present in high enough quantities to be measured. Recently, expression of *rpoS* was also detected in *Vibrio parahaemolyticus* under VNBC conditions (Coutard *et al.*, 2005).

In agreement with a previous report (Ruimy *et al.*, 1994), a large number of 16S rRNA copies was detected in exponentially growing *V. cholerae*. The number of 16S rRNA copies per live cell was between 50 000 and 110 000. These values are comparable to those observed for *Salmonella enterica* serovar Typhimurium (Fey *et al.*, 2004). They are also close to the number of ribosomes per bacterial cell described for *Escherichia coli*, namely 6700–71 000 depending on the growth rate and the physiological state (Gourse *et al.*, 1996). The number of mRNA copies for the *tuf* gene in exponentially growing cells (i.e. 200–370 copies cell^{-1}) was also similar to the data reported for highly expressed genes in other organisms (Karlin *et al.*, 2001; 2004). In contrast, an intense degradation of the *tuf* mRNA was observed both in the starvation and the VBNC states. This is consistent with the general metabolic decline in stressed cells, which is associated with a repression of highly expressed genes (Wai *et al.*, 1999) and the activation of proteins involved in repair or degradation processes (Desnues *et al.*, 2003).

Table 3. Number of RNA copies in *V. cholerae* cells in the VBNC state, starvation conditions and during exponential growth.

	After 9 days incubation in ASW at[b]		
Target RNA molecule[a]	15°C (starvation)	4°C (VNBC)	Exponential cells
16S rRNA	$(2.80 \pm 1.6) \times 10^3$	$(7.22 \pm 4.3) \times 10^2$	$(1.11 \pm 0.35) \times 10^5$
tuf mRNA	0.017 ± 0.01	0.053 ± 0.03	272 ± 120
rpoS mRNA	0.571 ± 0.50	0.267 ± 0.04	6.48 ± 0.81
relA mRNA	0.102 ± 0.08	0.335 ± 0.11	7.63 ± 3.9

a. Values are expressed as RNA copies per live cell. Live cells were measured as total number of particles retaining membrane integrity, according to the live/death staining.

b. Exponential cultures were washed two times with sterile ASW, diluted 100-fold in ASW, and further incubated at 4°C or 15°C for 9 days.

Interestingly, the differences observed between VBNC and starved cells were less striking. After 216 h (9 days) incubation at 15°C, cells in starvation exhibited a 3.9- and 2.1-fold increase in the levels of 16S rRNA and *rpoS* mRNA, with respect to bacteria in VBNC state (Table 3). On the other hand, the mRNA for *tuf* and *relA* were selectively increased in VBNC cells (3.0- and 3.2-fold respectively). Thus, it seems that VBNC cells might compensate, at least in part, for the reduction in ribosomal content by increasing the number of mRNA molecules for the elongation factors (i.e. *tuf*). The kinetic Q-RT-PCR analysis of the number of copies of *relA* mRNA present in *V. cholerae* cells entering the VBNC state and starvation showed that this increase is both, specific for VBNC cells, and matches the entrance in this state (Fig. 3D). It is important to highlight that, with the exception of the 16S rRNA, the number of copies of mRNA of the genes was always below one per cell, thereby suggesting that the transcription of these genes was shutdown in some members of the bacterial population. These cells could be dead, as has been shown for *E. coli* (Sheridan *et al*., 1998).

Application for environmental studies

Vibrio cholerae has developed different strategies to survive and persist in the environment. On the one hand, it can exploit higher organisms (e.g. mussels, snails, copepods, amoebae) as a reservoir for survival (Peterson, 2002; Reidl and Klose, 2002). On the other hand, bacterial long-term survival in water is rendered possible by entrance into either the VBNC (Ravel *et al*., 1995; Pruzzo *et al*., 2003) or the starvation state (Wai *et al*., 1999; Pruzzo *et al*., 2003) states. In contrast in other studies, only 9 days were required to the *V. cholerae* strain 3083 to reach the VBNC state (Chaiyanan *et al*., 2001; Pruzzo *et al*., 2003). This can be explained, at least in part, by the nutritional state of the population. When cells in the stationary phase are used, they are more resistant to low temperatures than bacteria in the exponential phase (Oliver *et al*., 1991; Weichart *et al*., 1992). Thus, we also evaluated the influence of the nutritional state of the population (i.e. exponential vs. stationary) in their response to cold shock. The obtained results (data not shown) demonstrated that bacteria in the stationary phase cells indeed maintain their culturability for longer times, as previously suggested (McDougald *et al*., 1998). Alternatively, the effect can be related to the specific strain employed in this study, as observed for *Vibrio vulnificus* (Fischer-Le Saux *et al*., 2002).

Although the levels of all tested mRNAs were reduced in VBNC cells when compared with those from exponentially growing bacteria, they have still been synthesized. Thus, key components of the cellular metabolism (i.e. *tuf* representing the protein synthesis and *relA/rpoS* the stress response) maintain their functionality. This is particularly true for the *relA* mRNA, which seems to be preserved in the VBNC, but not in the starvation state. As little as 8, 40 and 50, and 270 copies of *rpoS*, *relA*, *tuf* and 16S rRNA, respectively, were detected under our experimental conditions. This corresponds to approximately 30, 120, 10^3 and less than 1 VBNC cells under these conditions. This sensitivity of detection is in turn far bellow the average infective dose (i.e. 10^5–10^6 cells) for virulent *V. cholerae* [Bej *et al*., 1996; and FDA/CFSAN (http://vm.cfsan.fda.gov/~mow/chap7.html)]. Thus, the selected specific RNA could be used as molecular targets for either identification (i.e. rRNA) and/or determination of metabolic activity of *V. cholerae* cells in VBNC state present in environmental samples using the developed Q-RT-PCR methods, thereby facilitating the assessment of the bacteriological quality of drinking and/or bathing waters.

Experimental procedures

Bacterial strain and growth conditions

The *V. cholerae* strain 3083 El Tor was grown in Luria-Bertani (LB, Difco) broth at 37°C with shaking (150 r.p.m.). Bacterial growth was monitored by determining the optical density at 600 nm (OD_{600}). For establishing the number of viable bacteria (cfu), samples were serially in sterile ASW (Sigma), and 50 μl of the chosen dilution were plated on Trypticase Soy Agar plates (TSA, Difco) in triplicates. Plates were incubated at 37°C for 24 h. For calculation of the cfu per millilitre, only dilutions showing between 20 and 300 colonies were used.

Determination of total cell counts and live cell counts

The total number of cells per millilitre was determined using the blue fluorescent dye DAPI (Sigma, chemical no. D-9542), as previously described (Weinbauer *et al*., 1998). Live cells were measured as total number of particles retaining membrane integrity, according to the live/death staining. Viability determination was performed employing the BD™ cell viability kit (BD Biosciences, San Jose, CA) in a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA), according to the protocols recommended by the manufacturer. At least three replicates of total and live cell counts were carried out with each method and for each sampling point. Unspiked flasks were also processed as controls. Colony-forming units determination for samples with an expected cell number of < 100 cfu ml^{-1} was done by membrane filtration.

Generation of V. cholerae *cells in the VBNC and starvation states*

An overnight culture of *V. cholerae* was diluted 100-fold and inoculated into 100 ml of LB at 37°C with shaking (150 r.p.m.)

until it reached $OD_{600} = 0.5$. Bacteria were then pelleted by centrifugation (10 min, 3000 *g*), washed twice in sterile ASW and resuspended in ASW at a final OD_{600} of 0.25 (approximately 10^8 cells ml^{-1}). Six glass bottles containing 990 ml of sterile ASW were spiked with 10 ml of the bacterial suspension (approximately 10^6 cells ml^{-1}). Three bottles were incubated at 4°C and the other three at 15°C in the dark without shaking. Samples from every bottle were taken every 24 h to determine cfu ml^{-1}, viability and for RNA extraction (10 ml). For exponential cells, a volume of 1 ml was taken. Samples for RNA extraction were centrifuged at 13 000 *g* for 10 min and decanted, the remaining pellets were homogenized with 2 volumes of RNAprotect solution (QIAGEN) and further incubated for 5 min at room temperature to stabilize bacterial RNA (Bhagwat *et al.*, 2003). Reaction tubes were centrifuged at 13 000 *g* for 10 min, and the pellets were stored at −20°C until nucleic acid extraction. When the cfu ml^{-1} of cells at 4°C was below of 0.1, the bacteria were arbitrarily considered to be in the VBNC state.

Nucleic acid extraction

DNA and RNA extractions were performed with the DNeasy and RNeasy kits, as recommended by the manufacturer (QIAGEN, Hilden, Germany). For RNA extraction, the lysis was performed with 100 μl of lysozyme (500 μg ml^{-1}) for 5 min at room temperature. DNase (QIAGEN) treatment was performed for 30 min at room temperature.

Design of primers and standards for Q-PCR

All primers (Table 1) were purchased from MWG Biotech (Ebersberg, Germany). Targets for Q-PCR and Q-RT-PCR were the genes coding for the *V. cholerae* 16S rRNA, *tuf*, *rpoS* and *relA*, as well as the corresponding RNAs. To enable an accurate quantification of the RNA targets, RNA standards were generated. The RNA standards for each gene were generated as previously described (Fey *et al.*, 2004), using 58°C (16S rRNA, *tuf* and *rpoS* genes) and 56°C (*relA* gene) as PCR annealing temperatures. In brief, a second primer set was designed for each target gene in which (i) the primers were located up- and downstream of the sequences recognized by the first set (i.e. longer amplification product) and (ii) the forward primer contained the sequences of the T7 promoter. These primers were used to amplify the genomic DNA by PCR. The resulting products were purified with the PCR purification kit (QIAGEN) and subsequently transcribed *in vitro* with T7 polymerase using the Riboprobe System-T7 (Promega). This was followed by digestion with DNase I, purification using RNeasy (QIAGEN) and a second DNase I digestion on the purification column. The transcripts were then analysed by agarose gel electrophoresis (1% gels containing 0.65% formaldehyde) and quantified using the RiboGreen quantification kit from Molecular Probes.

Calculation of copy numbers

The numbers of copies of the Q-PCR standards were calculated by assuming average molecular masses of 340 Da for 1 nucleotide of single-stranded RNA. The calculation was done with the following equation: copies per nanogram $= (NL \times 10^{-9})/(n \times mw)$, were *n* is the length of the standard in base pairs or nucleotides, mw is the molecular weight per nucleotide, and NL is the Avogadro constant (6.02×10^{23} molecules per mol).

Two-step Q-RT-PCR and data analysis

The two-step Q-RT-PCR and data analysis were performed as previously described (Fey *et al.*, 2004). The reactions were performed on a RotorGene 2000 (Corbett) real-time PCR machine and the annealing temperatures employed were the same as described above. The cDNA was generated using the SybrGreen RT-PCR Reagents (Applied Biosystems) and random hexamers for priming, according to the instructions of the manufacturer. RNA standards were similarly treated. In each reverse transcription reaction, some RNA samples were not supplemented with reverse transcriptase to rule out DNA contamination. To generate a calibration curve, the serially diluted RNA standard (1000 pg to 0.001 fg) was quantified in each Q-PCR run. The calibration curves were generated by the Rotor-Gene software version 4.6. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the background or threshold (Ct value). The slope of each calibration curve was given into the following equation to determine the reaction efficiency: $\text{Efficiency} = 10^{-1/\text{slope}} - 1$. According to this, an efficiency of 1 means a doubling of product in each cycle. Using the calibration curve, the Rotor-Gene software calculated the initial number of target copies in the measured samples. From these values, the mean numbers of copies of mRNA for *tuf*, *rpoS*, *relA* and for 16S rRNA were calculated and expressed as number of copies per live cell.

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