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Quantitative RT-PCR Analysis of *Vibrio cholerae* Cells Entering the Viable but Non-Culturable State and Starvation in Response to Cold Shock

Narjol González-Escalona,^{1,2} Axel Fey,¹ Manfred G. Höfle,¹ Romilio T. Espejo,²

and Carlos A. Guzmán^{1*}

Vaccine Research Group, Division of Microbiology, GBF-German Research Centre for Biotechnology, Braunschweig, Germany,¹ and Laboratorio de Biotecnología, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Santiago, Chile²

Running title: Q-RT-PCR ANALYSIS OF *V. CHOLERA*E

* Corresponding author. Mailing address: Vaccine Research Group, Division of Microbiology, GBF-German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49-531-6181558. Fax: 49-531-6181411. E-mail: cag@gbf.de.

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-PCR (Q-RT-PCR). Bacterial cells entering the VBNC state showed a 154, 5.1x10³, 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.

1 INTRODUCTION

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

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

20 Bacteria that have reached the VBNC state are unable to grow in rich media, but still
21 maintain their viability (Lowder *et al.*, 2000; Pruzzo *et al.*, 2003). According to many studies,
22 pathogenic bacteria in the VBNC state retain their virulence (Baffone *et al.*, 2003; Lleo *et al.*,
23 2000; Oliver and Bockian, 1995). In fact, previous work has demonstrated that VBNC cells of *V.*
24 *cholerae* retain their pathogenic potential for a prolonged time (Chaiyanan *et al.*, 2001; Colwell,
25 1996; 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 analyzed (Carrol *et al.*, 2001; Hood *et al.*, 1986). 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 (Fischer-Le Seaux *et al.*, 2002; Lleo *et al.*, 2000; Sheridan *et al.*, 1998). 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-PCR (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). Since 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 quantitative reverse transcription-PCR (Q-RT-PCR). Studies were performed in parallel, to

1 assess bacterial viability and culturability of the *V. cholerae* cells. To obtain an accurate
2 determination of the RNA targets present in viable cells, we used RNA standards obtained by in
3 vitro transcription, according to our recently established method (Fey *et al.*, 2004). Our data
4 confirmed that key components of the metabolism are maintained functional in bacteria entering
5 the VBNC state and starvation, and that the newly developed Q-RT-PCR methods can be
6 successfully exploited for the identification of *V. cholerae* and the assessment of its metabolic
7 activity in environmental samples.

8 9 **RESULTS AND DISCUSSION**

10 **Assessment of *V. cholerae* survival in ASW.** To study the process of entering the
11 VBNC state and starvation, we performed a comparative kinetic analysis of bacterial cells that
12 were incubated in ASW using three different methods: (i) DAPI staining was used to determine
13 the total number of stained particles (i.e. bacterial cells), (ii) determination of the number of
14 CFU/ml by agar plating was done to establish the number of culturable cells able to grow in
15 conventional culture media (TSA), and (iii) the BD™ cell viability kit was employed to determine
16 by flow cytometry the number of viable cells (i.e., bacteria with intact membranes),
17 independently of their growth potential. When exponentially growing cells of the *V. cholerae*
18 strain 3083 were incubated in ASW at 4°C and 15°C, two different responses were observed
19 (Fig. 1A). After 24 h a rapid loss of culturability could be observed at both incubation
20 temperatures (i.e., 1 and 2 logs at 4 and 15°C, respectively). This loss of culturability correlated
21 with a fast reduction in bacterial viability (26%) with respect to the initial time for the cells
22 incubated at 15°C (Fig. 1A and B). On the other hand, when bacteria were incubated at 4°C for
23 24 h, only 4% of the cells have lost their membrane integrity. However, a continuous reduction in
24 bacterial viability was observed for cells incubated at both temperatures during the course of the
25 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 x 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 10⁶ 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 during 9 days until they reach the VNBC state and starvation (Fig. 3 A to D). The number of 16S rRNA molecules ranged from about 700 to 4,000 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

1 numbers of *rpoS* mRNA decreased also in parallel during the first 2 days, with higher values in
2 the 4°C samples (Fig. 3C). A slight recovery of *rpoS* mRNA was observed after 6 days with no
3 significant differences among the different temperatures. The copy number of *relA* mRNA
4 remained low for 6 days with no significant differences between incubation temperatures (Fig.
5 3D). After 9 days the VNBC cells showed a threefold increase in comparison to the starved cells
6 (Fig. 3D, Table 3).

7 The kinetic results given in Fig. 3 were compared with those from *V. cholerae* cells
8 growing exponentially (Table 3). For comparative reasons, the end points after 9 days for the
9 VNBC state and the starvation conditions were used. The number of RNA copies per live cell
10 was 6.5, 272, 7.6 and 1.1×10^5 for *rpoS*, *tuf*, *relA* and 16S rRNA in exponential growing cells
11 (Table 3). In contrast, bacteria entering the VBNC state showed 154, 5.1×10^3 , 24 and 23-fold
12 reduction in the number of copies of 16S rRNA and mRNA for *tuf*, *rpoS* and *relA*, with respect to
13 exponentially growing cells. Thus, the metabolic activities related to protein synthesis seem
14 extremely affected in VBNC cells (i.e., 16S rRNA and *tuf*). Regardless the elevated reduction
15 observed in the expression levels for all the RNAs tested, they were still present in high enough
16 quantities to be measured. Recently, expression of *rpoS* was also detected in *Vibrio*
17 *parahaemolyticus* under VNBC conditions (Coutard *et al.* 2005).

18 In agreement with a previous report (Ruimy *et al.* 1994), a large number of 16S rRNA
19 copies was detected in exponentially growing *V. cholerae*. The number of 16S rRNA copies per
20 live cell was between 50,000 and 110,000. These values are comparable to those observed for
21 *Salmonella enterica* serovar Typhimurium (Fey *et al.*, 2004). They are also close to the number
22 of ribosomes per bacterial cell described for *Escherichia coli*, namely 6,700 to 71,000 depending
23 on the growth rate and the physiological state (Gourse *et al.*, 1996). The number of mRNA
24 copies for the *tuf* gene in exponentially growing cells (i.e., 200-370 copies/cell) was also similar
25 to the data reported for highly expressed genes in other organisms (Karlin *et al.*, 2001; Karlin *et*

1 *al.*, 2004). In contrast, an intense degradation of the *tuf* mRNA was observed both in the
2 starvation and the VBNC states. This is consistent with the general metabolic decline in stressed
3 cells, which is associated with a repression of highly expressed genes (Wai *et al.*, 1999) and the
4 activation of proteins involved in repair or degradation processes (Desnues *et al.*, 2003).

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

18 **Application for environmental studies.** *V. cholerae* has developed different strategies
19 to survive and persist in the environment. On the one hand, it can exploit higher organisms (e.g.
20 mussels, snails, copepods) as a reservoir for survival (Peterson, 2002; Reidl and Klose, 2002).
21 On the other hand, bacterial long term survival in water is rendered possible by entrance into
22 either the VBNC (Pruzzo *et al.*, 2003; Ravel *et al.*, 1995) or the starvation state (Pruzzo *et al.*,
23 2003; Wai *et al.*, 1999) states. In contrast in other studies, only 9 days were required to the *V.*
24 *cholerae* strain 3083 to reach the VBNC state (Chaiyanan *et al.*, 2001; Pruzzo *et al.* 2003). This
25 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 versus 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 to 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 below 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

1 **Bacterial strain and growth conditions.**

2 The *V. cholerae* strain 3083 El Tor was grown
3 in Luria-Bertani (LB, Difco) broth at 37°C with shaking (150 rpm). Bacterial growth was
4 monitored by determining the optical density at 600 nm (OD₆₀₀). For establishing the number of
5 viable bacteria (CFU), samples were serially diluted in sterile ASW (Sigma), and 50 µl of the
6 chosen dilution were plated on Trypticase Soy Agar plates (TSA, Difco) in triplicates. Plates
7 were incubated at 37°C for 24 h. For calculation of the CFU per milliliter, only dilutions showing
8 between 20 and 300 colonies were used.

8 **Determination of total cell counts and live cell counts.**

9 The total number of cells per
10 ml was determined using the blue fluorescent dye DAPI (Sigma, chemical no. D-9542), as
11 previously described (Weinbauer *et al.*, 1998). Live cells were measured as total number of
12 particles retaining membrane integrity, according to the live/death staining. Viability
13 determination was performed employing the BD™ cell viability kit (BD Biosciences, San Jose,
14 CA) in a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA), according to the
15 protocols recommended by the manufacturer. At least three replicates of total and live cell
16 counts were carried out with each method and for each sampling point. Unspiked flasks were
17 also processed as controls. CFU determination for samples with an expected cell number of
18 <100 CFU ml⁻¹ was done by membrane filtration.

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

19 An overnight
20 culture of *V. cholerae* was diluted 100-fold and inoculated into 100 ml of LB at 37°C with shaking
21 (150 rpm) until it reached OD₆₀₀ = 0.5. Bacteria were then pelleted by centrifugation (10 min,
22 3,000 x g), washed twice in sterile ASW and resuspended in ASW at a final OD₆₀₀ of 0.25
23 (approximately 10⁸ cells ml⁻¹). Six glass bottles containing 990 ml of sterile ASW were spiked
24 with 10 ml of the bacterial suspension (approximately 10⁶ cells ml⁻¹). Three bottles were
25 incubated at 4°C and the other three at 15°C in the dark without shaking. Samples from every
26 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 x *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 x *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 analyzed by agarose gel electrophoresis (1% gels containing 0.65% formaldehyde) and quantified using the RiboGreen quantitation 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 = $(NL \times 10^{-9}) / (n \times mw)$, where 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 (C_t value). The slope of each calibration curve was given into the following equation to determine the reaction efficiency: 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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FIGURE LEGENDS

FIG.1. Survival of *V. cholerae* cells entering the VBNC and starvation states. (A) The total number of particles ml⁻¹ 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.

FIG. 2. Calibration curves generated using the RNA standards for *rpoS* (A), *relA* (B), *tuf* (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.

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.

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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	<u>TAATACGACTCACTATAGGG</u> CCTAGCTGGTCTGAGA	278-293	771	VCr001
	Vc 16S r-T7	ATGCAGCACCTGTCTCAGA	1031-1049		
<i>tufA</i> and B	Vc tuf f-T7	<u>TAATACGACTCACTATAGGG</u> TCATGAACAAGTGTGACATGGT	401-422	693	VC0321; VC0362
	Vc tuf r-T7	GCAATCAGGTCTACAACCATCT	1073-1094		
<i>rpoS</i>	Vc rpoS f-T7	<u>TAATACGACTCACTATAGGG</u> TGAGTGTGAGCAATAC	2-17	643	VC0534
	Vc rpoS r-T7	CGCAGCATCTTAGTGACATC	625-644		
<i>relA</i>	Vc relA f-T7	<u>TAATACGACTCACTATAGGG</u> TTGGTCAGCTCAAATGGGAA	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		
<i>tufA tufB</i>	Vc tuf f-st	ACTTATATTCCAGAGCCAGAGC	592-613	180	VC0321
	Vc tuf r-st	TGTACAGGTCGTTTTTACTGTC	750-771		
<i>rpoS</i>	Vc RpoS f 151	GCTTCTGCGAAAAGTCTTG	151-169	199	VC0534
	Vc RpoS R331	GTGCTAATCCTCGGTTGCT	331-349		
<i>relA</i>	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/>).

1

2 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
<i>tuf</i> mRNA	0.79 ± 0,01	84.6	50
<i>rpoS</i> mRNA	0.82 ± 0,07	84.3	8
<i>relA</i> mRNA	0.85 ± 0,02	83.6	40

3 ^a Reaction efficiencies are mean values of three or more determinations ± standard deviations.

4 ^b Number of copies.

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

2

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

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^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 2 times with sterile ASW, diluted 100-fold in ASW, and further incubated at 4°C or 15°C for 9 days.