Vibrio parahaemolyticus Strain ATCC 17802 Contains Two Transcriptionally Active 16S rRNA Genes and neither degrades preferentially in starvation

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Vibrio parahaemolyticus species type strain ATCC 18702^T contains two 16S rRNA gene (*rrs*) types in the 11 ribosomal operons. The differences between these two *rrs* types are concentrated in a variable stem loop and can be detected by the heteroduplexes produced after 16S rDNA PCR amplification of that region. In the present study we explored the existence of the same polymorphism in their 16S rRNA. We found that in exponentially growing cells the two *rrs* types were expressed. Furthermore, in cells under starvation conditions, although most RNAs were degraded, both 16S rRNA types were still present. These findings showed that both *rrs* types are being expressed in ATCC 18702^T and in starvation conditions neither type degrades preferentially.

Key words: polymorphism in 16SrDNA, *rrs*, Vibrio parahaemolyticus, 16S rRNA expression

1. Introduction

Many organisms have multiple copies of the rRNA gene that differ in nucleotide sequence, in some cases by up to 5% [6, 9]. Polymorphisms in 16S rDNA (*rrs*) are common in type collection strains and environmental strains of the genus *Vibrio* [10]. Polymorphisms in the *rrs* can be identified rapidly by monitoring heteroduplex formation after PCR amplification of 16S rDNAs [11]. When the *rrs* of a strain with multiple copies of the gene are PCR amplified, *rrs* amplification products with differing sequences form hybrids. These hybrids, or heteroduplexes, can be identified due to a retarded electrophoretic migration in polyacrylamide gels.

In recently examined strains of *Vibrio* [10], the polymorphic sites were concentrated in a recognized variable stem loop of the 16S rDNA, from nucleotides 440-496 (*Escherichia coli* annotation [12]). In some cases this region contained up to 83% of the total mutations. Specifically, the ATCC species type strain of *Vibrio parahaemolyticus* contains two different *rrs* types that differ in 10 nucleotide sites in the aforementioned region [10, 13]. Taking advantage of the fact that the polymorphisms are concentrated in a defined region, we monitored heteroduplex formation after PCR amplification of a short fragment containing this region [13], rather than PCR amplification of nearly the entire *rrs* genes [10]. A similar heteroduplex formation should be observed after RT-PCR of the 16S rRNA if both *rrs* types are transcriptionally active. In order to test if both *rrs* were transcriptionally active we conduct RT-PCR of the short region of total RNA extracted from *V. parahaemolyticus* cells grown under different experimental conditions

Ribosomal RNAs are considered to be "stable" but are highly degraded during carbon starvation, in stationary phase, and in slow growing conditions [14, 15]. Most bacterial metabolism studies have been conducted in cells in the exponential growth phase and little is known how the cells behave in natural environments [16]. Adaptation to non-growing states (*e.g.* starvation), as has been observed in bacteria present in oligrotrophic environments, requires that cells perform a large physiological adjustment [14, 16]. It has also been observed that in the carbon starvation state, it is essential for the cell that a fast and intensive degradation of the ribosomal proteins occurs along with degradation of the ribosomals RNAs [17, 18]. Nevertheless, a critical level of ribosomes should remain functional, otherwise the cells could not perform some necessary processes during nutrient starvation and would not be able to regain the capacity to return to fast growing conditions [16]. In survival studies of *V. choleare* conducted in artificial sea water at different temperatures, it had been observed that at 15ºC, cells display a starvation response and at 4ºC, cells convert to a viable but non cultivable state (VBNC) [19]. In both cases, a very sudden metabolic adjustment should occur in the cells. A similar situation could be present in *V. parahaemolyticus* when released to the water by the host [20]. For this reason, we wanted to test if starvation conditions induced degradation of one or both of the 16S rRNA. To accomplish this, cells of *V. parahaemolyticus* were spiked in an artificial sea water microcosm and incubated for 72 h at 3 different temperatures (4, 15 and 37ºC). The presence of the two 16S rRNA types was monitored by formation of heteroduplexes after RT-PCR amplification.

2. Materials and methods

2.1 Artificial Sea Water (ASW) preparation and culture conditions.

V. parahaemolyticus strain ATCC 17802 was used in this study and was directly obtained from the American Type Culture Collection, Manassas, VA. Sea Salts media (Sigma-Aldrich, Munich, Germany) was used for the creation of the artificial sea water (ASW) microcosm of a final concentration of 35 ppt and prepared as indicated by the manufacturer. ASW was filter sterilized (0.22 μm). The *V. parahaemolyticus* strain was grown overnight in LB at 37ºC with shaking (180 rpm). A dilution of 1:100 of the overnight culture was inoculated into fresh LB medium, using the same growth conditions until cultures reached an OD_{600} 0.5. Twenty ml of this exponential phase culture was centrifuged for 5 min at 4000 x *g* and the pellet was washed twice with sterile ASW. The final pellet was suspended in ASW at an OD_{600} 0.25 (ca. 10^8 cells/ml). Three bottles containing sterile ASW were spiked each with this suspension to a final concentration of approximately 10^6 CFU/ml. The bottles were incubated at 4, 15 and 37ºC, respectively for 72 h in the dark without shaking. Samples were taken at 24 and 72 h.

2.2 Extraction and relative quantification of the RNA.

For extraction of RNA, 1 ml was taken from the exponential and stationary phase cultures. For the ASW microcosms, 10 ml was samples. All samples were centrifuged 10 min at 4000 x *g*. The pellets were suspended in 0.5 ml of ASW then 1 ml of RNAprotect Bacteria Reagent was added (QIAGEN, Hilden, Germany). Samples were incubated for 10 min at room temperature and centrifuged at 5000xg for 10 minutes. Pellets were stored at -70ºC until processed (within 2 weeks). RNA extraction was carried out using the RNeasy Mini Kit (QIAGEN) per manufacturer's recommended protocol. For cell lysis, samples were incubated with 100 μl of Lysozyme (500 μg/ml) at room temperature for 5 min. DNase I (QIAGEN) treatment was performed at room temperature for 30 min; reaction mixture followed recommendations of the manufacturer. The RNA was eluted with a final volume of 30 μl of nuclease-free water. The extracted RNA was quantified using Ribogreen® (Molecular Probes, Inc., Eugene, OR) in a microtiter plate reader as described previously [21]. The RNAs were stored at -70ºC until analysis. The relative quantification was performed using the following formula: % RNA degradation = (RNA concentration of exponential phase – RNA condition tested) * 100. The value of the total RNA at exponential phase was considered 100 %. All RNA concentration values were calculated as fg/CFU. In exponential phase, stationary and starvation there were 3.4 *10^8, 1.2 *10^9, and 2 * 10^6 CFU/ml, respectively.

2.3 RT-PCR amplification of the 16S rRNA

A two step reverse transcription PCR **(**RT-PCR) reaction was employed for the amplification of the 16S rRNA. For the reverse transcription reaction the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, CA, USA) was employed, using random hexamers (3 μl). Five μl of RNA was used as template for the RT reactions. Total reaction volume was 15 μl and the final concentrations of the different components of the TaqMan® MicroRNA Reverse Transcription master mix (7 μl per reaction) was as follows: 1 mM dNTPs (with dTTP), 1 μl MultiScribe™ Reverse Transcriptase, 50 U/μL, 1X 10× Reverse Transcription Buffer, and 0.19 RNase Inhibitor (20 U/μL). RT conditions were performed according to manufacterer's instructions using a DNA Engine (PTC-200) thermocycler (MJ Research): an initial hold at 16ºC for 30 min, followed by 30 min at 42C, and a final enzyme inactivation step of 5 min at 85 ºC. PCR reaction conditions were as previously described, using 5 μl of cDNA obtained after the RT reaction as template [22]. Primers 357F and 518R were used for amplification of the 161 bp fragment containing the variable region of the 16S rRNA gene [13]. The PCR products were examined by electrophoresis in polyacrylamide gels (PAGE) and visualized by silver nitrate staining as described previously [23].

3. Results and discussion

3.1 Presence of the two 16S rRNA types in exponentially growing cells

Polymorphisms in the 16S rRNA of *V. parahaemolyticus* strain ATCC 17802^T were explored by examining the formation of heteroduplexes in the RT-PCR product of the rRNA as described in Materials and Methods. Figure 1A lane 2 shows the product of the RT-PCR of the 16S rRNA in exponential phase, performed with primers 357F and 518R [13]. The pattern obtained is indistinguishable from that obtained by PCR amplification of the genomic DNA (lane 1). This observation indicates that both types of 16S rRNA are transcriptionally active in the exponential phase of growth. Possession of two distinct types of transcriptionally active 16S rRNA genes has been previously described in the actinomycete *Thermomonospora chromogena* [6]. Furthermore, based on band intensity, it appears there is not a

selective regulation of the expression of the types of 16S rRNA (at least under these experimental conditions), in contrast with what has been observed in *E. coli* [24-26].

The possession of multiple ribosomal genes has been related to the capacity of some microorganisms to respond quickly to changes in nutrient rich media. Evidence also exists that suggests a differential regulation of the expression of operons in the same genome due to multiple ribosomal genes [25, 27]. The presence of multiple polymorphic ribosomal genes could confer some resistance to substances such as antibiotics that act at the 16S rRNA level (e.g. aminoglycosides) [28, 29].

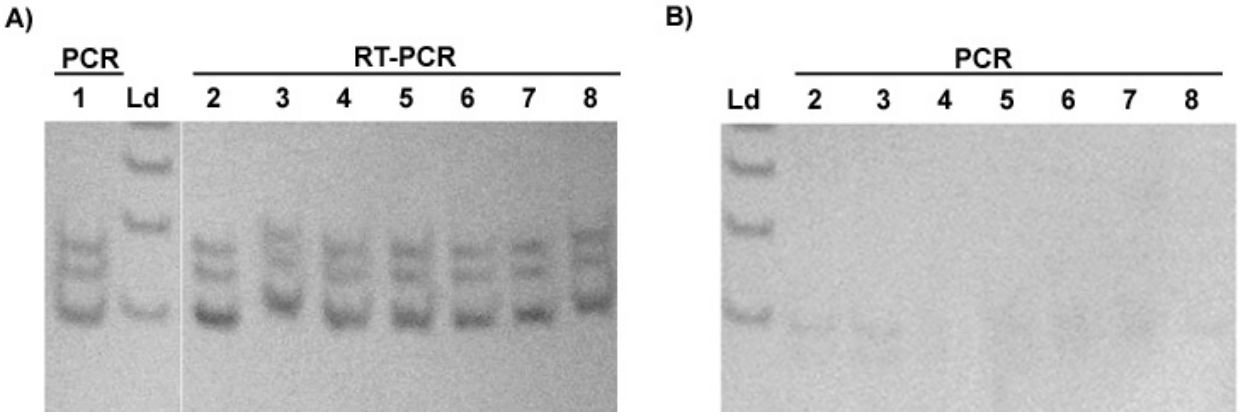

Figure 1. PAGE of the products obtained after PCR and RT-PCR amplification of the 16S rRNA from the total RNA extracted from *V. parahaemolyticus* ATCC 17802 under different experimental conditions. A) Lane 1, PCR amplification of *V. parahaemolyticus* DNA. Lanes 2-8, RT-PCR of RNA samples: 2 (RNA exponential phase), 3 (RNA stationary phase), 4 (RNA 4ºC, 24 h), 5 (RNA 15ºC, 24 h), 6 (RNA 37ºC, 24 h), 7 (RNA 4ºC, 72 h), 8 (RNA 15ºC, 72 h). B) Lanes 2-8, 16S PCR of RNA samples in the same order as above, without adding the RT enzyme. Ld corresponds to BenchTop 100bp DNA Ladder (Promega, Madison, WI, USA).

The total rRNA content in a cell depends on the rate of synthesis versus degradation. In *E. coli*, it is postulated that the rate of 16S rRNA synthesis from the different ribosomal operons may be differentially regulated depending on the bacterial growing conditions [25, 27]. However, how degradation of the 16S rRNA occurs in conditions of nutrient deprivation has not been thoroughly studied. In order to explore the whether a preferential degradation of the two types of 16S rRNA in *V. parahaemolyticus* ATCC 17802^T exists, a two-step RT-PCR from total RNA targeting the rRNA after nutrient starvation at different temperatures was conducted. Different temperatures were selected as temperature plays a very crucial role in bacterial growth and survival in the environment [19].

Three bottles of ASW were spiked with 10^6 cells/ml of *V. parahaemolyticus* from an exponential culture and incubated them at 4, 15 and 37ºC, respectively. We observed the level of total RNA fell quickly, within 24 h after inoculation, and after 72 h of incubation in ASW 96-98% of the RNA had been degraded (Table 1). Because the majority of total RNA composition is rRNA [13], it could be estimated that more than the 90% of the rRNA present in the exponential phase sample was degraded after 72 h in ASW. Similar outcomes have been reported for *E. coli* cells maintained in ASW and *Salmonella* strains in stationary phase, where approximately 50% of their 16S rRNA was degraded [16,15].

Table 1. Total RNA decrease in percent when *V. parahaemolyticus* cells entered nutrient starvation at different temperatures in comparison to the exponential phase.

% RNA [(RNA exponential – RNA nutrient starvation) x 100]						
	24 h in ASW			**72 h in ASW**		
Stationary phase	**4ºC**	**15ºC**	**37ºC**	**4ºC**	**15ºC**	**37ºC**
64	96	87	98	96	96	>98

In order to estimate the relative quantity of the two types of rRNA present in the ATCC strain of *V. parahaemolyticus*, the heteroduplex formation after RT-PCR was examined in the RNA samples extracted from cells

after time in starvation conditions. Figure 1A shows the heteroduplex patterns observed as a result of the RT-PCR of the rRNA from different experimental conditions (lanes 3-8). These patterns were indistinguishable from the heteroduplex patterns of *rrs* amplification and the RT-PCR of the rRNAs from exponential phase (Fig. 1A lane 1 and 2). A *rrs* PCR (no RT step) was conducted with the RNA samples to ensure no DNA remained in the samples (Fig. 1B). The results indicate that the 16S rRNA from the experimental conditions exhibit a similar polymorphism to that observed in the *rrs*. Therefore, degradation of the 16S rRNA was occurring in a non-selective way, and both types of rRNA are present in this strain in all conditions tested. In the case of slow growing cells of *E. coli*, it had been reported that the balance between the synthesis of the rRNAs and the ribosomal proteins is altered such that an excess of rRNA is produced and subsequently degraded to a great extent [15]. In this instance, we were not able to determine if the 16S rRNA observable at 72 h was synthesized *de novo* or was the 16S rRNA remaining from the initial spike of exponential cells. In order to address this interesting subject further investigation will be required.

Taken together, these results indicate that *V. parahaemolyticus* ATCC 17802 harbors two transcriptionally active *rrs* types and their degradation during nutrient starvation appear to occur in a non-selective way. In addition, this work brings to light some topics to be addressed further, such as: velocity of degradation and/or synthesis of rRNA in the different experimental conditions, particularly during simulation of environmental conditions.

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