

Detection of *Helicobacter pylori* associated with zooplankton

Luigina Cellini^{1,*}, Emanuela Di Campli¹, Rossella Grande¹, Soraya Di Bartolomeo¹,
Manuela Prenna², Matilde S. Pasquantonio², Luigi Pane³

¹Department of Biomedical Sciences, University 'G. D'Annunzio', Via dei Vestini 31, 66100 Chieti, Italy

²Department of Biology MCA, University of Camerino, 62032 Camerino, Italy

³Department of Experimental Environmental and Applied Biology, University of Genova, 16100 Genova, Italy

ABSTRACT: *Helicobacter pylori* was isolated from marine zooplankton, and characterized by standard microbiological tests, by PCR amplification of *vacA* and *cagA* gene fragments, and by comparative sequence analysis of the *vacA* PCR product. In a viable-but-non-culturable (VBNC) state, this isolate, as well as the reference strain *H. pylori* ATCC 43629, could be re-activated only when incubated in the presence of the marine copepod *Tigriopus fulvus*, and not in its absence. Isolate and type strain of *H. pylori* were found to be associated to the surface of *T. fulvus*, which supports speculations about a potential role of copepods in *H. pylori* survival and transmission.

KEY WORDS: *Helicobacter pylori* · Zooplanktonic organisms · Transmission · Seawater · Viable-but-non-culturable (VBNC) state · *Tigriopus fulvus*

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INTRODUCTION

Helicobacter pylori is a gastrointestinal pathogen that is one of the causative agents of gastritis and peptic ulcer diseases. It has also been described as a risk factor for gastric carcinoma (Go 2002, Suerbaum & Michetti 2002). The natural habitat for the microorganism is the human stomach, but it also needs to survive in other environments to become a life-long infection threat (Sasaki et al. 1999). *H. pylori* has been detected in dental plaque, in houseflies and in human and animal faeces (Grubel et al. 1997, Parsonnet et al. 1999, Kabir 2003), and a large number of studies report its presence in aquatic environments (Hulten et al. 1996, Baker & Hegarty 2001, Mazari-Hiriart et al. 2001, Park et al. 2001, Lu et al. 2002, Cellini et al. 2003). Water supplies contaminated by sewage containing fluids or faeces from infected people have therefore been considered as a potential source for *H. pylori* transmission (McKeown et al. 1999, Mazari-Hiriart et al. 2001, Park et al. 2001, Lu et al. 2002, Cellini et al. 2004).

Helicobacter pylori has been shown to react to environmental stress by modifying its morphology from

spiral to coccoid shape (Cellini et al. 1998). Among the coccoid cells, a portion is able to enter into the viable-but-non-culturable (VBNC) state, which is regarded as a temporary adaptation to unsuitable environmental conditions (Benaissa et al. 1996, Cellini 1996). In this VBNC state, bacteria are able to maintain their metabolic activity, their pathogenicity (Colwell & Huq 1994, Mizoguchi et al. 1998, Lleo et al. 2001) and, in the case of environmental changes, their ability to revert to a culturable state (Nilsson et al. 1991, Cellini et al. 1994, Colwell & Huq 1994, Lleo et al. 1998, Oliver 2005).

We have previously demonstrated the presence of *Helicobacter pylori* DNA in seawater and associated to zooplankton (Cellini et al. 2004). In that study, we speculated that the DNA belonged to intact bacteria able to survive in marine environments since dead/damaged bacteria and free DNA are rapidly degraded by the intensive activity of enzymes produced by pro- and eukaryotic cells (Hoppe 1991, Huston et al. 2000). The aim of the present study was to demonstrate the presence of intact cells of *H. pylori* in seawater and associated to zooplankton. The major objective of this research was therefore to isolate and characterize *H.*

*Email: l.cellini@unich.it

pylori cells from seawater samples and associated to zooplankton. An isolate and the type strain of *H. pylori* were subsequently used to study potential interactions with zooplankton. For this purpose, the marine copepod *Tigriopus fulvus* (Fisher 1860) found in rockpools of the Ligurian coast (Carli et al. 1993) was used as a model organism.

MATERIALS AND METHODS

Water sampling and processing. Water samples of 10 l were collected with a Niskin bottle at a depth of 5 m from the Adriatic Sea (42° 29' 41" N, 14° 12' 19" E), about 500 m from the coast of Pescara, Italy. Water samples were refrigerated at 4°C and processed within 4 h. They were first passed through a 200 µm mesh nylon filter (Idromar). Filters with retained organisms were transferred to sterile plastic containers, and covered with 10 ml of sterile seawater and sterile glass-beads. After detachment of organisms from the filter by a minishaker (IKA WORKS) at 600 rpm for 1 min, the samples were twice centrifuged with sterile seawater at 200 × *g* for 10 min to remove free bacteria, and the remaining organisms finally resuspended in 10 ml of sterile seawater.

The filtered water was subsequently passed through a 64 µm mesh nylon filter, which was subsequently treated as described above. The remaining filtered water was finally passed through 0.22 µm pore-size standard membrane filters (Pall Gelman Laboratory) to detect free cells of *Helicobacter pylori* and the filter was treated as above. From each sample, 3 equal aliquots were prepared before culture processing.

Isolation and characterization of *Helicobacter pylori*. From samples obtained on these different filters, 100 µl were directly spread on non-selective medium with 10% of defibrinate horse blood (Chocolate agar, CA, OXOID) supplemented with 1% of IsoVitaleX (Becton Dickinson), and on Campylobacter selective medium (CP, OXOID) and incubated at 37°C under microaerophilic conditions (Campy Pak Jar, OXOID) for 5 to 7 d. Plates were checked every day and potential colonies of *Helicobacter pylori* were sub-cultured on CA and CP under microaerophilic conditions. The remaining sea water sample was incubated under microaerophilic conditions for 10 d. Every day, 100 µl were spread both on CA and CP. Potential colonies of *H. pylori* were stained with modified Gram staining to ascertain the bacterial morphology. Curved, Gram-negative rods were subsequently tested for urease (Christensen's urea test, OXOID), catalase and oxidase activities characteristic for *H. pylori* (Spot test Oxidase Kit, Difco). Additional tests included susceptibility tests to clarithromycin, tinidazole, amoxicillin

and rifabutin (Toracchio et al. 2000). For molecular characterization, DNA was extracted using the QIAamp Tissue DNA isolation Minikit (QIAGEN), and used as a template for PCR detection of the *vacA* gene encoding the vacuolating cytotoxin and the *cagA* gene, the main determinant of *H. pylori* pathogenicity. For *vacA* gene detection, a 622 bp DNA fragment was amplified with oligonucleotide primers *vacA* 1sc (5'-CAA TCG TGT GGG TTC TGG AGC-3') and *vacA* 3as (5'-GCC GAT ATG CAA ATG AGC CGC-3') according to Monstein & Ellnebo-Svedlund (2002). For *cagA* gene detection, a 298 bp DNA fragment was amplified with oligonucleotide primers *cagA*-F (5'-TTA GAA TAA TCA ACA AAC ATC ACG CCA T-3') and *cagA*-R (5'-ATA ATG CTA AAT TAG ACA ACT TGA GCG A-3') according to Monteiro et al. (1996). PCR for *vacA* and *cagA* genes was carried out in a 2700 Thermocycler (Applied Biosystems) in a total volume of 25 µl, containing 50 ng of genomic DNA, 10× buffer, 3 mM MgCl₂, 20 pmol of each primer, 1.0 U of Amplitaq DNA polymerase (Applied Biosystems) and 250 µM of each deoxynucleotide triphosphate. *vacA* gene fragment amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 25 cycles at 94°C for 30 s, 51°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. *cagA* gene fragment amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Samples (5 µl) of PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel at 100 V for 1 h. Gels were stained with ethidium bromide and photographed. The *vacA* PCR products were purified by spin column QIAQuick (QIAGEN) and cycle-sequenced (on both strands) using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). DNA sequences were analyzed on an automated sequencer, ABI PRISM 310, version 3.4.1 (Applied Biosystems). In all analyses, *H. pylori* ATCC 43629 was used as the positive control and *Pseudomonas aeruginosa* ATCC 27853 as the negative control.

Preparation of VBNC cells. A *Helicobacter pylori* strain isolated from seawater samples containing zooplankton and *H. pylori* ATCC 43629 were plated on chocolate agar (CA) and incubated at 37°C for 7 d under microaerophilic conditions (Campy Pak Jar-OXOID). Colonies were harvested and resuspended in Brucella Broth (Biolife Italiana) supplemented with 2% fetal calf serum (BB; Becton Dickinson) and incubated under microaerophilic conditions at 37°C for 3 d. To obtain a synchronization of growth, broth cultures were passed through 0.8 µm pore-size membranes (Millipore). The filtered cultures were then incubated in BB under microaerophilic conditions at 37°C. Growth was

monitored by measuring the optical density at 600 nm (OD_{600}) (Biophotometer, Eppendorf). The number of culturable bacteria was assessed by plating 100 μ l of 10-fold concentrated broth cultures on CA and incubating them under microaerophilic conditions at 37°C for 3 d. The total number of microorganisms was determined using a Burkler chamber (Fortuna).

For the induction of the VBNC state, exponentially growing broth cultures of the isolate and the type strain of *Helicobacter pylori* were harvested at $OD_{600} = 0.3$, washed twice with PBS (pH 7.2) and resuspended in prefiltered and sterilized seawater (salinity 37 PSU) with a final cell density of about 10^6 colony forming units (CFU) ml^{-1} . These cultures were incubated under microaerophilic conditions at 37°C for up to 45 d, without agitation. Every 3 d, duplicate samples of 10 ml were filtered through 0.22 μ m pore-size membranes (Millipore). The filters were placed on CA and incubated under microaerophilic conditions at 37°C for 5 to 7 d. The viability of *H. pylori* VBNC cells was assessed by staining with the Live/Dead Kit (Molecular Probes). Living bacteria displaying green fluorescence were visualized with a Leitz Orthoplan epifluorescence microscope equipped with a 50 W mercury bulb and filter system (fluorescein, YOYO-1, SYTOX Green). For each determination 10 fields were counted. Experiments were carried out with VBNC *H. pylori* cultures, when the number of cultivable cells was <0.1 CFU ml^{-1} and all stained cells showed green fluorescence.

Co-cultivation of VBNC *Helicobacter pylori* with *Tigriopus fulvus*. A total of 10^3 VBNC cells of the isolate or type strain ATCC 43629 was suspended in several aliquots of 3 ml sterilized seawater microcosms (salinity 37 PSU) containing 20 individuals of *T. fulvus* each (10 males and 10 females). The prepared microcosms were kept at room temperature ($20 \pm 1^\circ C$) and checked directly after inoculation, and after 3, 6, and 24 h. Microcosms of *T. fulvus* without bacteria as well as microcosms of *Helicobacter pylori* without copepods were added as controls.

At each sampling, the contents of 2 entire microcosms were filtered through 200 μ m nylon filters. The filters were then washed twice with sterile seawater, covered with 3 ml of sterile seawater, and sonicated at 20% maximal output with a Labsonic 1510 sonicator (Braun) for 10 min to release copepods and bacteria from the filter. Of the recovered sample, 100 μ l, containing 2 individuals of *Tigriopus fulvus*, were spread either on campylobacter selective medium (CP) or CA. Plates were incubated under microaerophilic conditions at 37°C for 10 d. Bacteria in developing colonies were identified by biochemical tests as indicated before. Three colonies from each positive sample were randomly picked, transferred to CA, and incubated and characterized as indicated above.

Pellets of filtered aliquots from samples with *Tigriopus fulvus* that had been incubated either with or without *Helicobacter pylori* were also fixed in 10% buffered (NaH_2PO_4) formalin (Andersen et al. 1992).

Helicobacter pylori cells were detected by immunohistochemical staining performed on 5 μ m thick paraffin sections after proteolytic predigestion with Proteinase K ($20 \mu g ml^{-1}$) (Andersen et al. 1992). Sections were tested with the specific rabbit anti-*H. pylori* antibody Dako (DAKO test, Glostrup, dilution 1:25) in Dako diluent (Ashton-Key et al. 1996). For additional scanning electron microscopy analysis, the samples were treated in a solution of 2.5% glutaraldehyde with 0.1 M cacodylate buffer at pH 7.2 for 1 h and then washed with cacodylate buffer. After washing with PBS buffer (OXOID), the samples were post-fixed in 2% osmium tetroxide in cacodylate buffer, subsequently dehydrated in a series of graded ethanol in water solutions ranging from 50 to 100% and finally gold coated with sputter K-550X (Emitech). The samples were analyzed with a scanning electron microscope (LEO 435 Vp) at the following original magnifications: 100 \times (Fig. 3A); 1000 \times (Fig. 3A, inset); 250 \times (Fig. 3B) and 35 000 \times (Fig. 3B, inset).

RESULTS

In seawater samples, *Helicobacter pylori* colonies were only obtained in residues on 200 μ m filters but not in residues on 0.64 and 0.22 μ m filters. Initial identification of the environmental strains with urease, catalase and oxidase tests yielded positive results for 5 colonies which were also susceptible to drugs commonly used in *H. pylori* therapy. All colonies showed single PCR *vacA* and *cagA* gene fragments (Fig. 1) and identical DNA fingerprints, which suggested the isolation of a single microorganism (data not shown). Com-

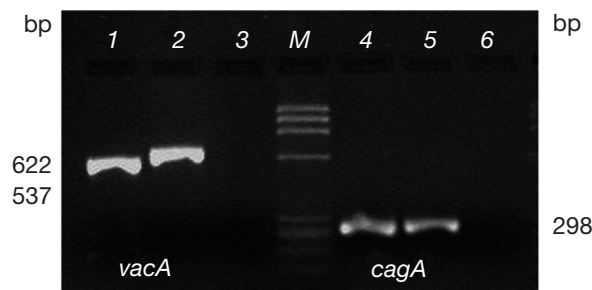


Fig. 1. *Helicobacter pylori*. Identification and characterization; agarose gel electrophoresis of PCR products of *vacA* and *cagA* genes. Lanes 1, 4: *H. pylori* isolate; Lanes 2, 5: *H. pylori* ATCC 43629; Lanes 3, 6: negative control (*Pseudomonas aeruginosa* ATCC 27853); M: marker $\Phi \times 174/HaeIII$

pared to the 622 bp *vacA* gene fragment of *H. pylori* ATCC 43629, the *vacA* fragment of the isolate was smaller in size (537 bp) (Fig. 1). Comparative sequence analysis of these fragments confirmed the identity of the environmental strain as *H. pylori* strain with a 89% similarity value (GenBank accession number: AY848858) to *H. pylori* ATCC 43629, more than 95% similarity to 5 other *H. pylori* strains, and on average 93% similarity to the remaining *H. pylori* strains deposited in DATA BASE National Center for Biotechnology Information (NCBI).

When cells of the isolate as well as of the type strain of *Helicobacter pylori* were in the VBNC state, cells could only be isolated after incubation in the presence of *Tigriopus fulvus*. Vegetative cells of *H. pylori* isolate were recorded after 3 and 6 h of incubation (10 and 150 CFU, respectively), and *H. pylori* ATCC 43629 was isolated after 6 and 24 h (90 and 115 CFU, respectively; Table 1). Each colony of *H. pylori* isolate and *H. pylori* ATCC 43629 was always positive for PCR-assisted detection of the *vacA* gene fragment and also for the *cagA* gene fragment (Fig. 2). Immunohistochemical analysis indicated the presence of *H. pylori* cells on the surface of the copepod (Fig. 3A); coccoid cells that resemble the morphology of *H. pylori* and attached to the chitinous carapace of the copepod were also detected by scanning electron microscopy (Fig. 3B).

DISCUSSION

In fractionated seawater samples, *Helicobacter pylori* could only be isolated from the fraction containing large zooplanktonic organisms; without zooplankton *H. pylori* cells could not be recovered from any

Table 1. *Helicobacter pylori*. Isolation after co-cultivation of viable-but-non-culturable (VBNC) cells with *Tigriopus fulvus* in artificial seawater microcosms. Values obtained averaging CFU on CA and CP agar of 2 replicate experiments. 0 h: time immediately after inoculation; CFU: colony forming units; C: contaminated sample; the presence of competing flora did not allow the detection of separate *H. pylori* colonies

	<i>H. pylori</i> CFU			
	Time (h) after inoculation of VBNC	0	3	6
<i>H. pylori</i> isolate	0	10	150	C
<i>T. fulvus</i>				
<i>H. pylori</i> ATCC 43629	0	0	90	115
<i>T. fulvus</i>				
<i>T. fulvus</i>	0	0	0	0
<i>H. pylori</i> isolate	0	0	0	0
<i>H. pylori</i> ATCC 43629	0	0	0	0

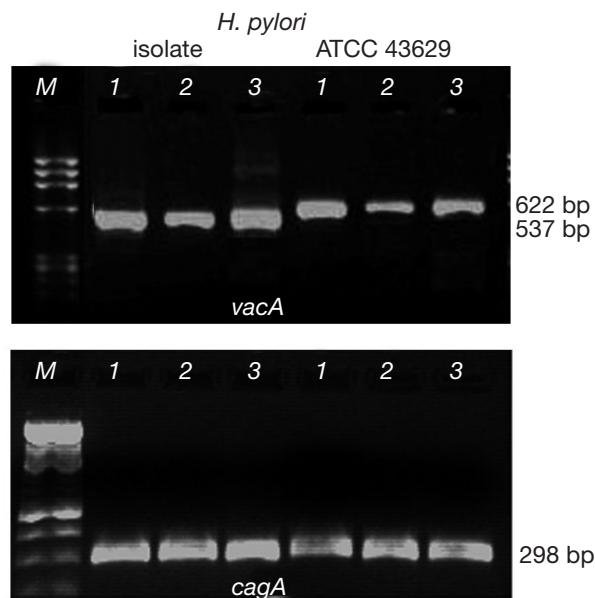


Fig. 2. *Helicobacter pylori*. Representative *vacA* and *cagA* genes detected before (Lanes 1) and after (Lanes 2, 3) co-cultivation with *Tigriopus fulvus*. Contact with *T. fulvus* was 3 h (Lane 2, left), 6 h (Lane 3, left; Lane 2, right) and 24 h (Lane 3, right). M: marker $\Phi \times 174$ /HaeIII

other fractions by growth-dependent detection protocols. These results, in part, confirm our previous observations on the presence of *H. pylori* associated to zooplankton (Cellini et al. 2004). Those nucleic acid-based detection protocols, however, also indicated the presence of *H. pylori* in seawater. Since nucleic acids are degraded rapidly and thus relatively unstable in seawater (Hoppe 1991), we assumed that these nucleic acids were part of intact cells (Cellini et al. 2004). Since these cells could not be retrieved by growth-dependent detection procedures, their adaptation to the environmental conditions might have resulted in the formation of VBNC cells. This phenomenon has been observed for several other organisms in the past (Nilsson et al. 1991, Colwell & Huq 1994, Lleo et al. 2001). Thus, a VBNC state for *H. pylori* might represent the most appropriate way for its survival under inadequate environmental conditions, such as unfavorable oxygen concentrations or non-permissive temperatures (Cellini et al. 2004). Since VBNC cells retain their virulence factors, as shown for *Vibrio parahaemolyticus*, these cells remain a potential risk in the environment (Coutard et al. 2005).

It has been suggested that a strategy for the persistence of *Enterococcus faecalis* cells in both lake and seawater is the formation of VBNC cells that adhere to plankton (Signoretto et al. 2004). Compared to free-living organisms, organisms associated with zooplankton have been shown to be different in morphology, size and metabolic activity (Tarsi et al. 2000). Zoo-

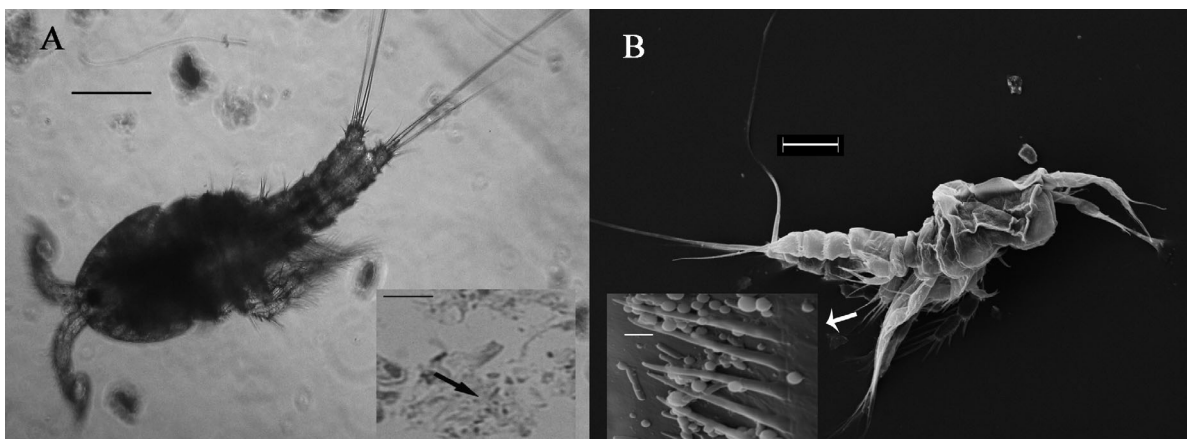


Fig. 3. *Helicobacter pylori*. Microscopic observation of interaction with *Tigriopus fulvus*. (A) *T. fulvus* co-cultivated with *H. pylori* embedded with formalin and (inset) microorganisms labelled with immunohistochemical staining (arrow); scale bar = 0.5 mm (inset = 10 μ m); (B) electronmicrograph of *T. fulvus* with (inset) coccoid *H. pylori* attached; scale bar = 0.1 mm (inset = 1 μ m)

plankton provides stable microhabitats for microorganisms in which cells are more likely to survive stressful conditions found in seawater than in free form (Huq et al. 1983, Colwell & Huq 1994, Winięcka-Krusnell et al. 2002). This assumption is supported by numerous studies on pathogenic bacteria other than *Helicobacter pylori*, in which the ecological relationship between epibiotic microorganisms and copepods results in protection from stress conditions (Signoretto et al. 2004, Zampini et al. 2005). Zooplankton can therefore be a reservoir for pathogenic bacteria such as *Vibrio* spp., including *V. cholerae* (Colwell & Huq 1994). *Vibrio* spp. mineralize chitin-containing substrates (Pruzzo et al. 1996, Tarsi et al. 2000), and thus have been found to be associated with chitin-containing copepods such as *Tigriopus fulvus* (Pruzzo et al. 2003, Zampini et al. 2005).

The identification of the environmental strain obtained by using the species-specific target gene *vacA* and the confirmatory DNA sequencing indicated that the isolate was a true *Helicobacter pylori* strain. Comparative analysis of the *vacA* sequence of *H. pylori* isolate with all bacterial genomes present in DATA BASE never found an alignment with organisms different to *H. pylori*. In particular, the *H. pylori* isolate *vacA* showed a deletion of 85 bp with respect to *H. pylori* ATCC 43629. The difference in the amplified fragment size obtained in the present study confirms the high genetic variability in *H. pylori* cells (Suerbaum 2000, Cellini et al. 2003). In the aquatic environment, *H. pylori* isolate retains the *cagA* gene, emphasizing the role of seawater in the diffusion of virulence genes representing a serious public health risk. In a previous study, it was suggested that a genomic rearrangement occurs among the microorganisms in freshwater environments, with a partial or total loss of

the *Cag* pathogenicity island where the *cagA* gene is localized (Mazari-Hiriart et al. 2001). In our study, the isolated strain was able to conserve this pathogenicity gene, representing a potential reservoir of *cagPAI* genes. Since the *cagPAI* island could be transferred to other environmental bacteria, such as *Aeromonas* spp. (Datta et al. 2003), the fate of *H. pylori* in the aquatic environment cannot be underestimated in a potential dispersion of virulence genes among organisms. Future studies will therefore have to address the question of dispersal mechanisms and gene transfer between organisms. We are currently investigating the presence and the potential role of plasmids in our marine isolate, which could enhance its ability in zooplankton adhesion and virulence gene transfer.

Acknowledgements. This study was supported in part by grant Cofin 2000, from the MIUR (Ministero Istruzione, Università e Ricerca), Rome, Italy. We thank Tonino Traini for his help on the microscopic observation of plankton.

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