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Role of Polyphosphate Kinase Gene (*ppk*) for Survival of *Vibrio cholerae* O1 in Surface Water of Bangladesh

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Abstract: Polyphosphate provides a substitute for ATP and energy source when phosphorus is a limiting resource in nature. The present study focuses on the role of polyphosphate for the survival of *Vibrio cholerae* in the aquatic habitats as an autochthonous bacterium. The survival advantages of polyphosphate of *V. cholerae* O1 having (wild type) and lacking (mutant) polyphosphate kinase (*ppk*) gene in surface water and with *Anabaena variabilis* were compared by cultural, Direct Fluorescent Antibody (DFA) and polymerase chain reaction methods in natural water microcosms. The microcosm's water was prepared by filtering and physicochemical parameters were also investigated by standard methods. The results revealed that both fresh and saline water, the wild type strain enhanced survival in cultural conditioned than *ppk* mutant strain. However, Fluorescent Antibody Direct Viable Counts (FADVC) and Polymerase Chain Reaction (PCR) results noted both strains have the equal survival strategy in viable but nonculturable state (VNC). In conclusion, it could be hypothesized that the polyphosphate inclusion body might keep cultivable and survivable at low phosphate natural environment of the aquatic bacterium.

Key words: Polyphosphate, Bangladesh, microcosm, viable but non-culturable, *Vibrio cholerae*

INTRODUCTION

Cholera caused by *Vibrio cholerae* particularly serogroups O1 and O139 containing toxin gene is endemic or epidemic in poor sanitation conditions in Asia, Africa and Latin America. The bacterium is presently established as autochthonous in aquatic environments and already reported to survive long periods in estuarine and brackish waters (Huq *et al*., 1984; Islam *et al*., 1999). *Vibrio cholerae* has been associated with a blue-green alga, *Anabaena variabilis* and provide a microenvironment for long-term survival in the aquatic environment (Islam *et al*., 1999). Mucinase, a soluble haemagglutinin/protease (HA/protease) present in *V. cholerae* degrade the mucilaginous sheath of *Anabaena* spp. (Islam *et al*., 2002; Mizanur *et al*., 2001). *V. cholerae* synthesizes much of inorganic polyphosphate (Ogawa *et al*., 2000) which, could also act as an ATP Substitute and energy source for the survival (Jahid *et al*., 2006). The viable but non-culturable (VNC) denotes the state in which, the cells cannot be detected by standard culture on enriched agar media, although remaining viable and capable of resuscitation under favorable conditions, described in numerous human pathogens like *Escherichia coli* (Xu *et al*., 1982), *V. cholerae* (Colwell *et al*., 1996), *V. vulnificus* (Oliver *et al*., 1991). Various viability assays have been successfully employed to detect the presence of VNC cells, such as direct viable count (Kogure *et al*., 1979), florescence microscopy using various stains (Rodriguez *et al*., 1992) and more recently molecular genetics methods (Warner and Oliver, 1998). Microcosms are limited form of model for *in situ* conditions, but it permits the controlling of selected environmental parameters as well as replication of conditions in and between experiments. Thus, microcosms are well suited for evaluating effect of environmental parameters on populations of bacteria.

In the present study, we evaluated the role of polyphosphate for the survival of *V. cholerae* in the aquatic environment establishing as the autochthonous fresh and estuarine bacterium.

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MATERIALS AND METHODS

Organisms and culture conditions: Two strains of *V. cholerae* O1 were used for the study. *V. cholerae* C6709-1, biotype El Tor, serotype Inaba, a toxigenic strain containing the *ppk* gene isolated in 1991 from a patient in Peru as the wild type strain. The other strain (AJB37) was an isogenic *ppk* gene deleted mutant of *V. cholerae* O1, El Tor Inaba. Both the strains were kindly provided by J.A. Benitez, Morehouse School of Medicine, Atlanta, USA (Jahid *et al*., 2006). These strains were reconfirmed as *V. cholerae* O1 by culture, morphological, biochemical and serological tests following standard procedures (Tison, 1999). Strains were frozen and stored at -70°C in T_1N_1 broth containing 15% glycerol broth. *A. variabilis* used in these experiments was obtained from the Department of Botany, University of Dhaka, Bangladesh.

Sources and preparation of water: The fresh and saline water were collected from Dhonagoda river of Matlab, Chadpur and river of Karnaphuli, Chittagong, Bangladesh, respectively. The water were then filtered through 0.22 μm millipore filter and added to the microcosms.

Preparation of inoculums: For each independent experiment, one cryovial was thawed and inoculated in Taurocholate Tellurite Gelatin Agar (TTGA) medium (Difco) and when required, streptomycin was added at a concentration of 50 μg mL^{-1} at 37°C for 24 h. The strains were then inoculated in LB broth containing streptomycin incubated at 37°C for 18 to 24 h in shaker at 120 rpm and then cells were harvested by centrifugation and washed three times with Phosphate Buffered Saline (PBS). After the final wash, the cell pellet was suspended in PBS and final concentrations were adjusted to ~10^4 CFU mL^{-1} in microcosms.

Microcosm experiments: Laboratory microcosms consisting of filtered sterile natural water in Erlenmeyer flasks which allowed for replication of experimental units and conditions between experiments were prepared in the following manner. Erlenmeyer flasks (2000 mL) were cleaned with acid hydrochloride and rinsed 10 times with double-distilled, de-ionized water. For each strain of *V. cholerae* O1 both wild type and mutant, microcosms were prepared in two conical flasks (2000 mL). In flask A, 1000 mL filtered water were added and 1 mL inocula of *V. cholerae* wild type were also added each flask so that final concentration of inoculated *V. cholerae* became ~10^4 CFU mL^{-1}. In flask B, 1000 mL water with mutant strains was inoculated. In flask C and D, filtered water and *V. cholerae* were added with 1 g of *A. variabilis*.

Thus prepared, the microcosms were incubated under static condition at 25°C in the laboratory. Sampling for culture and detection of VNC *V. cholerae* O1 started 30 min after preparation of the microcosms which was considered as the 0 h reading and continued at various time intervals up to one year. Three kinds of samples were taken each time: i) *A. variabilis* from flask C and D, ii) water from flask C and D where *A. variabilis* was floating and iii) water from flask A and B . At each sampling, 0.1 g *A. variabilis* was taken and homogenized by a tissue grinder (StedFast Stirrer Model 300, Fischer Scientific) following procedures described by Huq *et al*. (1984).

Measurement of physico-chemical parameters: The salinity, dissolved oxygen (DO), pH and turbidity were measured using portable meters (HACH Conductivity Meter, Cat. No. 51800-18; HACH Portable Dissolved Oxygen Meter, Cat. No. 51850-18; Sension TM6, CO, USA and Orion Portable pH Meter, Cat. No. 210 A; Orion Research, MA, USA; Portable Microprocessor Turbidity Meter, HI, 93703, Hanna Instruments, Hungary). Nitrite and phosphate were determined in a Skalar-SAN-plus autoanalyser (Skalar Analytical BV, Breda, the Netherlands) according to standard spectrophotometric methods for seawater (Kattner and Becker, 1991).

Culture of *V. cholerae* O1: Serial dilutions of *A. variabilis* and water were prepared in normal saline and the counts of cultural *V. cholerae* O1 were monitored using TTGA plates containing streptomycin in duplicate following drop plate technique (Hoben and Somasegoran, 1982). The plates were incubated at 37°C for 18-24 h and then the characteristic *V. cholerae* O1 colonies were counted. After incubation, the bacterial counts were derived from the number of individual colonies and expressed as CFU mL^{-1} or g. The arithmetic mean was calculated from the colony counts of duplicate plates. The counting of any sample was discontinued after failure to recover *V. cholerae* O1 on two consecutive samplings following enrichment technique. Each experiment was repeated two times.

Direct fluorescent antibody-direct viable count (FADVC): Detection of *V. cholerae* O1, formaldehyde-preserved samples were processed by using cholera DFA kits (Cholera-DFA kit; New Horizon Diagnostics Corp., USA) (Hasan *et al*., 1995). Briefly, samples were incubated in the dark at room temperature for 6 to 8 h in the presence of (0.025%) yeast extract and (0.002%) nalidixic acid (Kogure *et al*., 1979). After incubation, samples were fixed with 4% formaldehyde. Then the samples were processed for direct fluorescent microscopy technique. In brief, 5 μL

samples were placed on a glass slide and a drop of 100% ethanol was added. After the samples were air dried, the samples were incubated with a lyophilized monoclonal antibody-fluorescein isothiocyanate conjugate (Cholera-DFA kit; New Horizon Diagnostics Corp., USA), containing the monoclonal antibody to the O antigen of *V. cholerae* O1, in a dark moisture chamber at 37°C for 30 min. Samples were observed immediately or stored at -20°C and observed within 1 to 2 days by epifluorescence microscopy (Model BH-2, Olympus, Japan) at a maximum excitation wavelength of 490 nm. Cholera-positive control and cholera negative control provided by the manufacturer (New Horizon Diagnostics Corp.) were used as positive and negative control, respectively. The *V. cholerae* O1 was counted following the procedure described earlier (Hasan *et al*., 1994).

DNA extraction: DNA was extracted from microcosm of *A. variabilis* and water described protocol by Murray and Thompson (Rivera *et al*., 2003) with some modification. Briefly, a 1 mL aliquot of a plankton or water sample was homogenized, centrifuged and suspended in TE buffer (10 mM Tris-HCl; 1M EDTA;pH-8.0) treated with 10% SDS and freshly prepared proteinase K and incubated at 50°C for 1 h. After treatment with lysozyme, proteinase K and ammonium dodecyl sulfate, DNA was extracted with cetyltrimethylammonium bromide and phenol-chloroform-isoamyl alcohol, concentrated with isopropanol and resuspended in TE buffer.

Detection of *ctx*A gene by PCR: DNA extracted from water and plankton samples was used as the template to detect *V. cholerae* O1. Simplex PCR were performed for the confirmation of *ctx*A gene among the microcosm water and *A. variabilis* at different days after formation of nonculturable state according to Singh *et al*. (2002). The primers for *ctx*A gene, *ctx*A1 (5'-CTCAGACGGGATTTGTTAGGCACG-3') and *ctx*A2 (5'-CGGGCAGATTCTAGACCTC CTG-3').

RESULTS

Biochemical characteristics: From the biochemical results it was found that all the cultural and biochemical, serologically, both the wild type and mutant were same (data not shown) and the differences of the strains only the accumulation of polyphosphate. All other characters remained same.

Physicochemical parameters variables in fresh and estuarine water: Table 1 shows the physicochemical parameters of fresh and estuarine water after filtration which affects the survival of *V. cholerae* O1 in natural water. Except pH, all others parameters were higher in saline water rather than fresh water. The phosphate concentrations were 0.035 and 0.93 μM mL^{-1} for fresh and saline water, respectively.

Table 1: Physicochemical parameters of microcosms from Matlab and Karnaphuli, Bangladesh

Parameters	Fresh water (Matlab)	Saline water (Karnaphuli)
pH	7.880	7.120
DO (mg L^{-1})	4.280	6.550
Salinity (ppt)	<0.010	19.300
Turbidity (NTU)	7.000	38.400
PO_4 (μM mL^{-1})	0.035	0.930
NO_2 (μM mL^{-1})	0.213	2.062

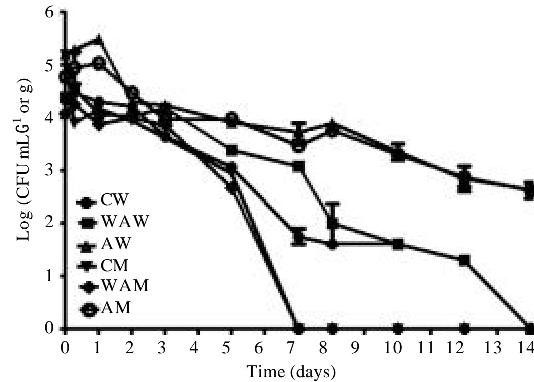

Fig. 1: Survival curve of *V. cholerae* O1 of fresh water microcosm in Bangladesh. The values shown are the Mean±SEM of three independent experiments, CW: Counts of wild type from water of flask A containing only water, CM: Counts of mutant from water of flask B containing only water, AW: Counts of wild type with *Anabaena* sp. of flask C containing water with *Anabaena* sp., AM: Counts of mutant with *Anabaena* sp., of flask D containing water with *Anabaena* sp., WAW: Counts of wild type in water of flask C containing water with *Anabaena* sp., WAM: Counts of mutant in water of flask D containing water with *Anabaena* sp.

***V. cholerae* O1 viable counts:** Figure 1 shows the survival of wild and mutant type of *V. cholerae* O1 in culturable state in fresh natural water microcosms. The initial count was ~$\log_{10}4.0$ CFU mL^{-1} or g and gradually decreased and it was found that the wild type survived 12 days in water whereas mutant survived only 5 days in water. The same trend was found in the fresh water on which *A. variabilis* was floating and wild type was cultured more than 12 days whereas mutant was only 5 days. The longest survival and multiplication of both wild and mutant were

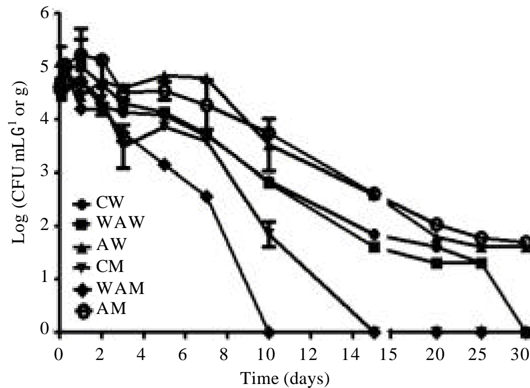

Fig. 2: Survival curve of *V. cholerae* O1 of saline water microcosm in Bangladesh. The histogram values shown are the Mean±SEM of three independent experiments. CW: Counts of wild type from water of flask A containing only water, CM: Counts of mutant from water of flask B containing only water, AW: Counts of wild type with *Anabaena* sp. of flask C containing water with *Anabaena* sp., AM: Counts of mutant with *Anabaena* sp. of flask D containing water with *Anabaena* sp., WAW: Counts of wild type in water of flask C containing water with *Anabaena* sp., WAM: Counts of mutant in water of flask D containing water with *Anabaena* sp.

observed in association with *A. variabilis*. The counts increased after 24 h and slowly decreased and remained culturable using LB with streptomycin antibiotic up to 14 days. Both wild and mutant showed the same survival patterns with *A. variabilis*.

Figure 2 shows the viable pattern of both the *V. cholerae* O1 in the natural saline water microcosms. It was noted the same survival pattern of fresh water microcosms but significantly higher survival was demonstrated at the condition. In control saline water the wild type cultured up to 25 days whereas mutant strain was not cultivated after 7 days. The mutant strain was cultured up to 10 days whereas wild type strain survived 25 days in water on which the *A. variabilis* was floating. Both the strains survived more than 30 days in associated with *A. variabilis* and no survival difference was observed for wild and mutant strains.

Enumeration of VNC *V. cholerae* O1 by FADVC: The representative figure of DFA counts of *V. cholerae* O1 with *A. variabilis* showed presence of bacterium with *A. variabilis* with fluorescence color (Fig. 3).

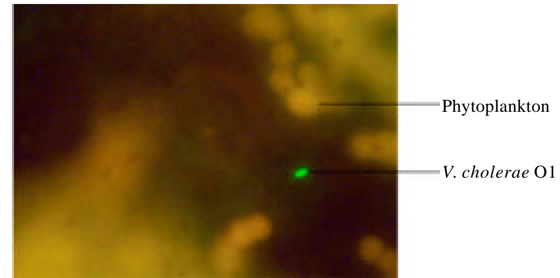

Fig. 3: Representative figure of *V. cholerae* O1 detection by DFA from phytoplankton samples in fresh water

Table 2: Fluorescent antibody direct viable counts (FADVC) (log cells mL^{-1} or g) of fresh water microcosms in Bangladesh

Days	CW	CM	WAW	WAM	AW	AM
0	4.631	4.666	4.903	4.763	4.642	4.714
1	4.859	4.467	4.765	4.740	4.703	4.881
2	4.532	4.672	4.607	4.727	4.823	4.825
3	4.496	4.685	4.518	4.624	4.905	4.756
5	4.402	4.373	4.330	4.547	4.635	4.754
8	3.913	3.806	3.635	4.296	3.356	3.699
10	3.699	3.699	3.502	3.678	3.259	3.718
15	2.958	3.259	3.259	3.657	2.958	3.503
30	2.657	3.202	3.202	2.833	3.135	2.657
45	2.356	2.958	2.657	2.657	2.958	2.356
360	2.356	2.356	2.356	2.657	2.834	2.356

CW: Counts of wild type from water of flask A containing only water, CM: Counts of mutant from water of flask B containing only water, AW: Counts of wild type with *Anabaena* sp., of flask C containing water with *Anabaena* spp. AM: Counts of mutant with *Anabaena* sp. of flask D containing water with *Anabaena* sp., WAW: Counts of wild type in water of flask C containing water with *Anabaena* sp., WAM: Counts of mutant in water of flask D containing water with *Anabaena* sp.

Table 2 shows FADVC of wild type and mutant *V. cholerae* in fresh natural control water and in association with *A. variabilis* and in water on which the *A. variabilis* was floating. The initial count of *V. cholerae* O1 in water was 4.6 $\log_{10}$ cells g^{-1}. After 5 days, the count did not decrease and then decreased and remained 2.35 $\log_{10}$ cells g^{-1} even after one year. Multiplication of *V. cholerae* was observed in association with *A. variabilis*. The initial FA count of *V. cholerae* with *A. variabilis* was 5.0 $\log_{10}$ cells g^{-1}. After 10 days, the count did not decrease and remained 4.2 $\log_{10}$ cells g^{-1} and then slowly decreased but remained constant even after one year.

Table 3 shows FADVC of wild type and mutant *V. cholerae* O1 in association with *A. variabilis* in saline water on which the phytoplankton was floating and in natural saline water. The same trend was noted like the fresh water microcosm. It was noted that ~3 log10 cells g^{-1} was found even after 1 year.

Table 3: Fluorescent antibody direct viable counts (FADVC) (log cells/mL or g) of saline water microcosms in Bangladesh

Days	CW	CM	WAW	WAM	AW	AM
0	4.571	4.640	4.259	4.666	4.496	4.135
3	4.301	3.913	4.417	4.149	4.176	4.334
7	4.176	4.000	4.527	3.612	4.328	4.421
10	3.771	3.900	4.453	3.913	4.231	4.320
15	3.259	3.754	4.237	3.737	4.038	4.010
20	2.958	3.754	4.356	2.958	3.436	4.010
310	3.135	3.356	4.000	2.959	2.958	3.848

CW: Counts of wild type from water of flask A containing only water, CM: Counts of mutant from water of flask B containing only water, AW: Counts of wild type with *Anabaena* sp. of flask C containing water with *Anabaena* spp. AM: Counts of mutant with *Anabaena* sp. of flask D containing water with *Anabaena* sp., WAW: Counts of wild type in water of flask C containing water with *Anabaena* sp., WAM: Counts of mutant in water of flask D containing water with *Anabaena* sp.

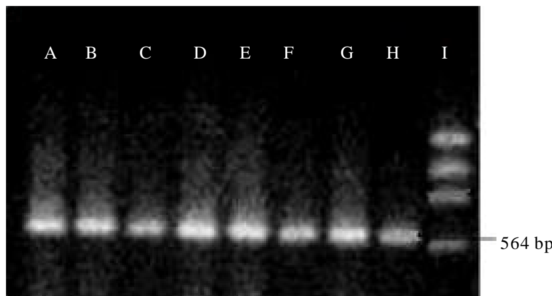

Fig. 4: Agarose gel electrophoresis of *ctxA* PCR product, Lane-A: Water of wild type from fresh water microcosm, Lane-B: Water of mutant from fresh water microcosm, Lane-C: Water of wild type where *A. variabilis* was floating, Lane-D: Water of mutant type where *A. variabilis* was floating MW, Lane-E: Associated *Anabena* sp. from wild type, Lane-F: Associated *Anabena* sp. from mutant type, Lane-G: Positive control of *V. cholerae* O1 and Lane-H: Positive control of *V. cholerae* O139, Lane-I: Marker DNA

Detection of *ctx*A gene by PCR technique: Figure 4 shows that the *ctx*A gene of *V. cholerae* is present in water and algal samples after 12 months of being nonculturable in microcosms. The 564 bp amplicon was observed from the samples examined indicating the amplification of target sequence of *ctx*A gene of both wild type and mutant. The results showed that both wild and mutant types *ctx*A gene was positive in every samples tested. The presence of *ctx*A gene indicated the presence of VNC cells even after one year.

DISCUSSION

Cholera is a dreadful diarrhoeal disease from the time immemorial in Bangladesh and other countries of the world. *V. cholerae* is considered as a most frightening agent that causes cholera globally. This global impact occurs since *V. cholerae* has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (Islam *et al*., 1993). *V. cholerae* is free-living in surfaces of freshwater, oceanic and estuarine environments.

It is well established that phosphorus and nitrogen play crucial roles in the ecology of aquatic ecosystems (Benitez-Nelson, 2000). Phosphorus has been proposed to be the most common cause of eutrophication in freshwater lakes, reservoirs, streams and the headwaters of estuaries, while nitrogen is believed to be the key mineral nutrient controlling primary production in the ocean (Benitez-Nelson, 2000). It is likely that in an environment in which phosphorus is limiting, bacteria capable of synthesizing large polyphosphate stores could have a competitive advantage. Inorganic polyphosphates are ubiquitous in nature and are found in all organisms examined (Wood and Clark, 1988).

In the present study, the wild type strains of *Vibrio cholerae* O1 EL tor Inaba (C6709-1) containing *ppk* gene and its isogenic mutant AJB37 which have the same biochemical behavior and also have same survival pattern in VNC state in both fresh water and saline water microcosms but different pattern were found in both control water and the water where *A. variabilis* was floating. The wild type strains survived longer than mutant in water might have been due to the ability of production of polyphosphate and accumulation in the body of the cells and used the polyphosphate as the ATP sources when the other nutrients were depleted. In this comparative study, the mutant strains did not able to store polyphosphate and did not survive as like wild type. Both the wild type and mutant strains were survived equally in association with *A. variabilis* as both the strains might have the ability to produce mucinase which helped to penetrate the mucilaginous sheath of *A. variabilis*. Both the strains degraded the mucin and used the degradation products as nutrients. As a result, both the strains persisted equally in culturable state in associated with *A. variabilis*. The mutant strains did not become VNC as they get the nutrient from *A. variabilis*.

Survival was observed up to 12 months by direct fluorescent technique because it has been found in previous studies *V. cholerae* can survive years in microcosms (Islam *et al*., 1999). The VNC *V. cholerae* has been monitored in microcosms up to 25 months and found to be present inside the mucilaginous sheath of *A. variabilis*. (Islam *et al*., 1999). The present study indicated that the production of enzymes particularly mucinase might help *V. cholerae* to use the degradation

products of mucilagenous sheath as nutrients in absence of phosphate as nutrient. Studies have shown that mucilaginous sheaths of blue-green algae are composed of carbohydrates and proteins (Fogg and Pattnaik, 1966) and that filtrates of blue-green algae contained various sugars and amino acids such as galactose, glucose, glutamic acid, alanine, serine, threonine, glycine, tyrosine, valine, leucine, phenylalanine and aspartic acid (Walsby, 1974).

Figure 1 and 2 show that both the wild and mutant *V. cholerae* O1 survived longer as VNC state than culturable state but we did not know how long they survived as VNC state. Therefore, after continuous sampling for 26 days, we made occasional sampling to detect the VNC *V. cholerae* from control water, water in which the *A. variabilis* was floating as well as the *A. variabilis* itself. Finally, the VNC state by FA technique was confirmed by more sensitive techniques like PCR. By using this technique, it was found that both strains were detected from all the samples up to 12 months (Fig. 4). Studies have demonstrated that microbial cells may enter into a viable but nonculturable state due to effect of various environmental conditions e.g., temperature, pH, salinity etc. (Colwell and Huq, 1994; Huq *et al*., 1984). Non-culturable *V. cholerae* O1 might have significant impact on cholera transmission as it could be as culturable form in their stools if ingested in volunteers as VNC state (Colwell *et al*., 1996). So, the nonculturable state of *V. cholerae* O1 determined in the microcosms associated with blue-green algae may pose problems as it may be able to convert into culturable form if ingested to human.

CONCLUSION

However, we conclusively proved that *ppk* gene is responsible to keep in culturable conditions of *V. cholerae* O1 in the aquatic environment as important features of ecology. As the polyphosphate is responsible to be nonculturable, it could be hypothesized that it has also the role even low concentrations present in environments.

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