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Bacterial Persistence in Aquatic Environments: A Study of Green Fluorescent Protein (GFP)-Tagged *Escherichia coli* in Laboratory Microcosms

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ABSTRACT

Background and Objective: In-depth knowledge of survival potential and survival patterns of wild-type and genetically engineered *E. coli* in aquatic systems is scarce. The study was designed to investigate the relative persistence and survival of transformed *E. coli* DH5α and environmental isolates. **Materials and Methods:** Three different types of microcosms were set up and inoculated with GFP-transformed *E. coli*. Samples were collected at regular intervals and the survival and growth of the organisms were monitored by viable counts using GFP as a cellular marker. Survival curves were prepared to visualize bacterial persistence trends over time. **Results:** The obtained results suggested different survival patterns of *E. coli* in different microcosms. The survival of *E. coli* in filter-sterilized water samples was found greater than that in untreated water. A sharp decrease in the count of *E. coli* cells in untreated water, compared to that of filter-treated and/or autoclaved water suggests that the survival of *E. coli* in natural water might be limited due to deprivation of nutrients or biological interactions, such as competition and predation. **Conclusion:** Transformation of GFP in *E. coli* did not show a significant effect on the persistence in the laboratory microcosm underscoring the potential utility of GFP-tagged *E. coli* as a model organism for studying bacterial survival and distribution dynamics.

KEYWORDS

Escherichia coli, green fluorescent protein (GFP), transformation, microcosm, survival

INTRODUCTION

Survival has been defined as the maintenance of viability under adverse environmental circumstances[1,2]. After being discharged from the host to the environment, *E. coli* encounters adverse nutritional and physicochemical shocks or stresses that influence significantly its survival[3]. Survival has been shown to be affected by numerous interacting factors, which include competition[4], predation[5], nutrient availability[6], temperature[7], osmotic conditions[8], radiation[9], pH[10], presence of antibiotics, algal toxins, heavy metals and other physicochemical factors[11,12]. Although *E. coli* is an enteric organism, previous research indicated its survival in aquatic environments[13]. Due to the difficulty of observing a particular species in mixed

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populations, the survival and persistence of intestinal bacteria like *E. coli* in the environment are frequently poorly known. On the other hand, due to the existence of starving or damaged cells or possibly "viable but nonculturable" cells, the use of culture-based, conventional enumeration techniques alone may dramatically underestimate the number of viable cells[14,15]. A unique method of monitoring such cell types in the environment can be accomplished by incorporating of certain cellular markers into the test bacteria, an example being a bioluminescent marker. It is now possible to identify and gauge the activity of recombinant bacteria in the environment by creating a bacterium with the bioluminescence trait[16,17]. In this context, research has been carried out to investigate the use of a plasmid-borne green fluorescent protein (GFP) gene as a marker for monitoring the survival of *E. coli* in natural environments. The GFP can function as a reporter protein and *in situ* cell marker in a wide variety of prokaryotic and eukaryotic cells[18,19].

The GFP is expressed two to three times faster in *E. coli* and exhibits eighteen-fold greater fluorescence intensity than native GFP. The main benefits of the GFP-tagged technique over the lux-based system, which needs an aldehyde substrate for bioluminescence[17], include its species independence and absence of need for any substrates or cofactors. Thus, GFP is a useful marker system that may be quickly identified by epifluorescence microscopy[20], spectrofluorometry, direct fluorescence measurement and fluorescence colony counting. Additionally, all growth phases, including those with starvation, are recognized by the green fluorescent phenotypes, allowing for nondestructive detection[21].

The survival capacity of the *E. coli* strains in the environmental water can be studied by correlating the persistence and recovery of *E. coli* cells in laboratory microcosm experiments. Suitable laboratory systems that model particular environments to monitor the fate of genetically engineered bacteria in such environments before initiating field experiments are commonly termed microcosms[22]. Microcosms can serve as standard test systems that can be adapted to a variety of organisms and environmental conditions[22] and therefore, are very useful and straightforward in examining microbial ecological processes[23-26].

Due to the restrictions placed on the release of these engineered microorganisms into the environment, studies in microcosm model systems are vital for studying their survival. However, by using a range of different microcosms rather than a single design, the results may prove more meaningful for predicting how strains will survive in an open environment[3].

The objectives of this study were to investigate the survivality of GFP-tagged *E. coli* in aquatic microcosms in the laboratory setting and to assess the potential utility of GFP-tagged *E. coli* as a model organism for studying bacterial survival and distribution in aquatic ecosystems.

MATERIALS AND METHODS

Study area: This work has been conducted in the Environmental Biotechnology Laboratory of the Department of Microbiology, University of Dhaka. This study takes 12 months (March, 2008 to February, 2009) from inception to completion.

Preparation of competent cells for transformation: A new overnight culture of 100 mL of the chosen strain of *E. coli* DH5a collected from the institutional repository was added to 100 mL of LB broth and it was then cultured at 37°C to an OD_{600} of 0.5 to 0.6. Cells were extracted by centrifugation (750-1000 x g, 12-15 min, 4°C) after being frozen on ice for 10-15 min. After being pelleted, the cells were resuspended in 0.25 culture volume of ice-cold 0.1 M $CaCl_2$ solution and held on ice for 30 min before being harvested as usual. Cells were kept on ice for at least 2 hrs (ideally overnight, as the cells are most competent the day after storage). Glycerol was added to the mixture to reach a final concentration of 20% for storage[20].

Transformation of 2 L of DNA solution (pGFP) and one hundred microliters of competent cells was combined and incubated on ice for 20-30 min. The cells were promptly cooled on ice for 2 min after being heat shocked at 42°C for 1 min. With the addition of 1 mL of LB broth and an hour of incubation at 37°C, they were given time to recover. The converted cells were plated onto a selective LB agar plate containing 0.5% arabinose and ampicillin (100 g L^{-1}) antibiotic.

Determination of transformation efficiency: The transformation efficiency was calculated by dividing the number of colonies by the amount (µg) of DNA added.

Laboratory microcosm set-up: Seven 250 mL conical flasks were taken for microcosm study. Each flask contained 100 mL of pond water (collected from the pond in front of Dhaka University Shahidullah Residential Hall). Inoculum consisted of 200 µL of actively growing cells of about 10^7 cells/mL concentration resuspended in normal saline. The following three different types of microcosms were used for this study:

- Microcosm with non-sterile pond water containing either transformed DH5α or transformed environmental strains
- Microcosm with filter-sterilized (using 0.22 µm millipore filter) pond water containing either transformed or untransformed parent strains of DH5α (without pGFP)
- Microcosm with filter-sterilized (using 0.22 µm millipore filter) pond water containing either transformed or untransformed parent environmental strains of *E. coli* (without pGFP)

Two additional microcosm experiments were set up using transformed *E. coli* DH5α in autoclaved distilled and autoclaved pond waters.

One flask of non-sterile pond water was taken as a control where no inoculum was added to check for the presence of any non-GFP conferred green fluorescent colonies. Treatments were performed in triplicate and microcosm flasks were regularly kept in a shaking incubator to provide proper aeration and agitation.

Counting interval: Water samples were collected from microcosm flasks on days 0, 1, 2, 3, 4, 7, 10, 15, 30 and 50. Samples were diluted appropriately and GFP- fluorescent cell counts (in case of transformed inocula) and total viable cell (in case of parent inoculum) counts were performed[27] on LB agar medium supplemented with ampicillin and arabinose as well as non-supplemented LB agar respectively. Finally, a plot of time intervals in days versus log of CFU mL^{-1} of water sample was prepared for each type of microcosm.

Statistical analysis: For the growth study, the viable counts were expressed as Log_{10} CFU mL^{-1} and plotted against incubation time (hr) to obtain the growth curves for each sample. Data were analyzed by the Gompertz equation to produce fitted growth curves. All statistical tests were considered significant at a confidence level of 95% ($p<0.05$).

RESULTS

Transfer of green fluorescent protein containing plasmid (pGFP) to *E. coli* strains: The control strain *E. coli* DH5α and one avian strain of *E. coli* were transformed with GFP. The transformation efficiency of *E. coli* DH5α was found to be 5.0×10^4 CFU μg^{-1} and the transformation efficiency of the environmental *E. coli* strain was 1.0×10^4 CFU μg^{-1}.

Survival potential of transformed *E. coli* in different types of microcosms: In the case of non-sterilized pond water containing microcosms, the initial inoculum of both *E. coli* DH5α and the environmental strain was about 10^6 CFU mL^{-1}. No significant change in the viable count was observed in the case of *E. coli*

DH5α until after 24 hrs (Fig. 1) whereas, a sharp decrease in the viable count of the environmental strain was observed from the very beginning. A gradual decrease in the cell count was observed in both types of microcosms with time intervals and after 10 days of inoculation, no pGFP-containing cells were observed.

The survival of *E. coli* in filtered water samples was found relatively better than that in untreated water. In filter-sterilized pond water, the reference DH5α strain and the environmental *E. coli* strain demonstrated a consistent survival pattern (Fig. 2 and 3). Initial inoculum of *E. coli* DH5α in filter-sterilized pond water was about 10^6 CFU mL^{-1} and initially there was no significant change in the viable count within 48 hrs. Then a slow, gradual decrease in the cell count was observed with time intervals, the untransformed strain showing somewhat better survival (Fig. 2). On day 20, 100 µL water was spread on arabinose arabinose-containing plate but no fluorescent colony was observed. Then on day 22, 10 mL of the water sample was filtered using a 0.22 µm millipore filter and the filter paper was placed on an arabinose-containing plate. However, no fluorescence was observed.

On the other hand, the initial inoculum of environmental isolate was about 10^5 to 10^7 CFU mL^{-1} and it showed a gradual decrease in the cell count with time intervals from the very beginning (Fig. 3). Due to heavy contamination, further counting after 10 days became impossible.

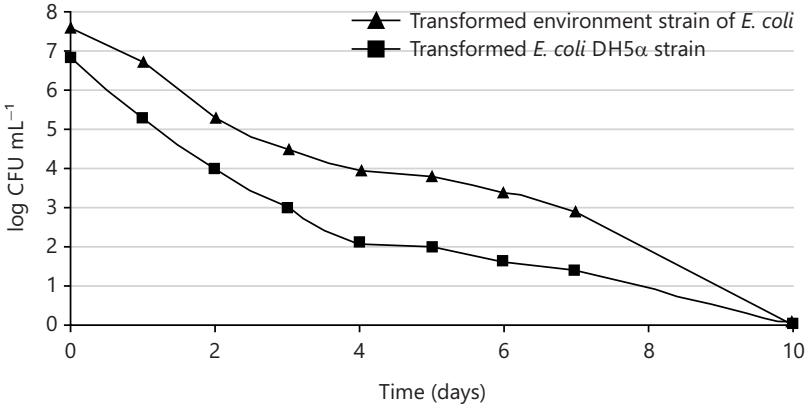

Fig. 1: Graph showing the culturability of both DH5α and environmental strains of *E. coli* in non-sterilized pond water

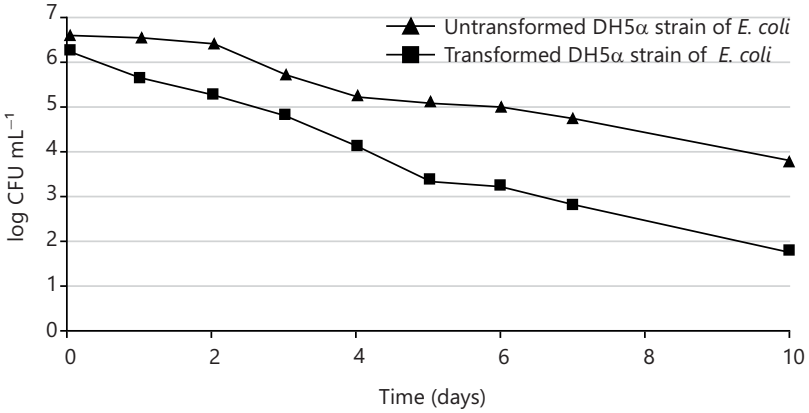

Fig. 2: Graph showing the culturability of both transformed and untransformed DH5α strains of *E. coli* in filter-sterilized pond water

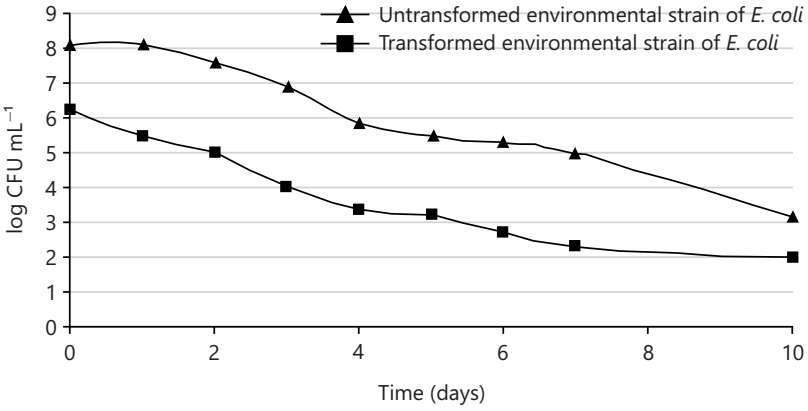

Fig. 3: Graph showing the culturability of both transformed and untransformed environmental strains of *E. coli* in filter-sterilized pond water

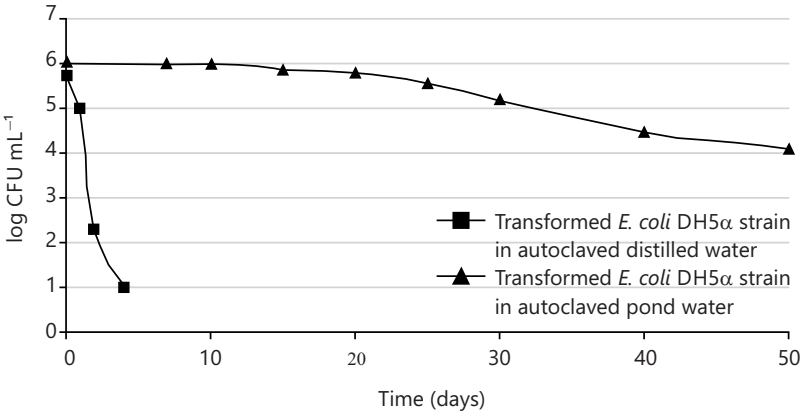

Fig. 4: Graph showing the culturability of transformed DH5α strain of *E. coli* in both autoclaved distilled water and autoclaved pond water

Microcosm with autoclaved pond water showed the maximum growth response of the *E. coli* DH5α strain. The initial inoculum was 10^5 CFU mL^{-1} and the setup was continued for 48 days, a gradual fall in cell count was observed with time. On the other hand, the growth response was minimal in autoclaved distilled water where the cultivability was observed for only 7 days (Fig. 4). After that, no green fluorescent colony was observed in the plates. The spread plate done on nutrient agar media did not reveal any colony.

The control flask was regularly checked by plating with arabinose and without arabinose-containing media. No green fluorescent colonies were observed during the period of investigation.

DISCUSSION

The microcosm model described in the present study is a simple but useful way to study the fate and survival of genetically modified *E. coli* in aquatic environments. In order to assess long-term bacterial viability in nutrient-limited aerobic settings and mixed microbial communities with diverse types of water, the study successfully transformed pGFP into *E. coli* DH5 and an environmental strain.

The maintenance of GFP fluorescence in cells under various settings and the connection between GFP fluorescence and cell viability were studied using microcosms containing sterilized water samples. GFP was highly stable under nonselective conditions and produced brightly fluorescence colonies which were easily detectable with a standard epifluorescent microscope[27,28].

The present study revealed that there was a significant difference between the survival of *E. coli* in sterilized and non-sterilized pond water. In this study, the survival of DH5α and the environmental strain of *E. coli* was observed for 10 days in non-sterile pond water. Growth of transformed *E. coli* cells was dramatically reduced in the non-sterilized microcosms compared to the sterilized ones, suggesting that indigenous microorganisms play an important role in the die-off of *E. coli* in the environment. After 10 days no single GFP colony was observed in the plates.

The obtained result revealed the plausible impact of indigenous predators and competitor microbes in the environment. Thus, the survival of *E. coli* in nature might be limited by biological interactions. *Escherichia coli* in untreated water quickly perished as a result of competition and predation[29,30]. Other genetically modified strains used in soil experiments with similar outcomes have been reported[31-33]. It has frequently been demonstrated that biotic variables reduce the lifespan of bacteria put into different aquatic environments. These factors include competition for nutrients[34] and the possibility of bacteriophage infection[35]. Several studies have shown the importance of protozoans in controlling bacterial populations[36,37]. In a study of the factors affecting the survival and growth of introduced bacteria in natural water bodies, it has been reported that the growth of the introduced species was limited due to the availability of carbon and sometimes nitrogen and phosphorus[38,39].

Transformed cells did not survive in filter-sterilized water for more than 10 days. One reason behind this may be the toxins and bacteriophages that might not be removed by filtration and thus, might play an adverse role in the survival and growth of *E. coli*. According to reports, autoclaving has advantages over filtering that may be attributable to one or more of the following factors: (i) Destruction of bacteriophages, (ii) Inactivation of thermolabile toxic substances like antibiotics and (iii) Increase in nutrients that are readily available[30]. Unfortunately, because of the contamination problem, counting of transformed environmental strains was not continued after 10 days.

In autoclaved distilled water, comparatively short survival of transformed *E. coli* DH5α strain indicated that the unavailability of nutrients in autoclaved distilled water and the probable release and accumulation of toxic materials in the cells might limit their survival. However, DAPI (4′,6-diamidino-2-phenylindole) staining of water samples would have explained whether the cells remained viable but nonculturable state.

On the other hand, the highest rate of survivability was observed in autoclaved pond water. The numbers of CFU mL^{-1} in this water treatment declined steadily and were surviving until 48 days and onwards. The persistence of *E. coli* in autoclaved water suggests its survival pattern in nature that might be limited by biological interactions, such as competition with the natural microbial flora[40]. Another significant finding from this experiment is that autoclaving improves the dissolved organic carbon's liability, potentially creating a better carbon supply for *E. coli* than regular pond water. Bacteriophages are destroyed by autoclaving and thermolabile harmful chemicals like antibiotics are rendered inactive[41,42]. In this study, there was a consistently significant difference in the survival of *E. coli* in sterilized and non-sterilized water. However, research carried out by Banning *et al*.[27] obtained no consistent significant difference between the survival of *E. coli* in sterilized and non-sterilized water. The reason behind this might be they performed the investigation under anaerobic conditions. A similar result was also found in previous studies[41,42].

This study provides insights into the survivality of GFP-tagged *E. coli* in laboratory aquatic microcosms, which can help in understanding the fate and behavior of bacterial populations in natural aquatic environments. The implications of these findings extend beyond the laboratory setting, as they provide insights into the behavior and potential ecological consequences of GFP-tagged *E. coli* in natural aquatic environments. The results of this study can help in designing better strategies for managing bacterial

contamination in aquatic environments, such as drinking water reservoirs, swimming pools and wastewater treatment plants. The study may stimulate further research into the molecular mechanisms underlying bacterial survival in aquatic environments, leading to a better understanding of microbial ecology and evolution.

CONCLUSION

Our findings revealed that GFP-tagged *E. coli* exhibited a persistent presence in the laboratory microcosms over time. In this experiment, no significant difference was observed among the survival patterns of the parent and transformed strains of *E. coli*. The uniformity of such response indicates that the presence of pGFP did not adversely affect the fitness of the host strains. Furthermore, our study highlights the importance of monitoring and managing bacterial populations in aquatic systems to mitigate potential risks associated with microbial contamination.

SIGNIFICANCE STATEMENT

Escherichia coli is a ubiquitous bacteria with potential aquatic transmission capability. In-depth knowledge of survival potential and survival patterns of wild-type and genetically engineered *E. coli* in aquatic systems is scarce. This study employed water microcosms to avoid issues related to the release of genetically engineered *E. coli* into the environment and to mimic real aquatic environmental conditions. Transformed *E. coli* showed comparable survival in microcosms compared to untransformed *E. coli*, indicating genetically modified pathogens can survive in aquatic environments. This study shed light on the possibility that enteropathogenic *E. coli* may survive in aquatic environments for a prolonged period.

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