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Use of Green Fluorescent Protein To Monitor Survival of Genetically Engineered Bacteria in Aquatic Environments

LAURA G. LEFF* AND ADAM A. LEFF

Department of Biological Sciences, Kent State University, Kent, Ohio 44242

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Many methods for detecting model genetically engineered microorganisms (GEMs) in experimental ecosystems rely on cultivation of introduced cells. In this study, survival of *Escherichia coli* was monitored with the green fluorescent protein (GFP) gene. This approach allowed enumeration of GEMs by both plating and microscopy. Use of the GFP-marked GEMs revealed that *E. coli* persisted in stream water at higher densities as determined microscopically than as determined by CFU enumeration. The GFP gene did not negatively impact the fitness of the host strain.

A variety of methods have been used to monitor the survival and fate of genetically engineered microorganisms (GEMs). Selectable markers, such as antibiotic resistance, have been used as a method for rapid enumeration of introduced cells and differentiation from native organisms (e.g., see reference 3). Many previous studies which have documented survival and mortality of introduced bacteria have relied on culturing and conventional selection regimens to quantify the bacteria of interest (e.g., see references 3, 4, 9, and 10). This approach is imperfect in that, over time, many bacterial species lose their ability to be cultured (8). Studies which have relied on culturing of introduced cells to document extinction may have documented the decline in culturability of the introduced bacteria or measured the frequency of cellular injury (2a). These introduced cells may have persisted throughout the study in a viable, nonculturable state, thus giving misleading results.

The purpose of this study was to investigate use of a plasmid-borne green fluorescent protein (GFP) gene as a marker for monitoring survival of GEMs. The GFP gene has recently been described and used as a reporter gene (1). The advantage of this approach is that it allows evaluation of the survival of GEMs in two ways: by plating to examine culturable cells and by microscopy to examine the total number of GEMs. In this way, even if GEMs lose culturability during the course of the experiment, their total abundance can be monitored directly by microscopic examination of samples. Unlike the *lux* system, GFP detection does not require addition of a substrate (5). The GFP method may not work under all environmental situations, such as anaerobic conditions. Both the *lux* and GFP approaches have advantages over traditional antibiotic resistance markers, which rely on culturing for detection (3).

Escherichia coli JM109 was electroporated to enable uptake of the pGFP cDNA vector (GenBank accession number U17997; replication origin from pUC; copy number, 500; Clontech, Palo Alto, Calif.). The resulting transformants exhibited ampicillin resistance (MIC, 20 μg/ml) and appeared bright green when viewed with a hand-held UV light (wavelength, 365 nm; model UVGL-48 Mineralight lamp [UVP, Upland, Calif.]).

E. coli was introduced into 100-ml flasks of stream water, and its abundance was monitored on days 0, 1, 2, 3, and 7 after inoculation. Inocula consisted of actively growing cells resuspended in sterile stream water. Stream water was collected from Bixon Creek, a low-order tributary of the Mahoning River in northeastern Ohio. Treatments were performed in triplicate, and flasks were shaken at 100 rpm at 24°C, a temperature which is similar to river water temperature maxima. Samples were removed at each interval and plated on Luria agar (10 g of NaCl, 10 g of Difco tryptone, 5 g of BBL yeast extract, and 10 g of Difco Bacto Agar in 1,000 ml of deionized water) with or without ampicillin. The plates were incubated for 48 h at 37°C, and total and green fluorescent colonies were enumerated. Water samples were also preserved with 0.2% Formalin and refrigerated until microscopic examination.

For microscopic enumeration, samples were filtered through a black polycarbonate 0.2-μm-pore-size filter and mounted with type FF immersion oil. The number of green fluorescent cells was enumerated with a Nikon epifluorescence microscope and a GFP filter set (Chroma Technology Corp., Brattleboro, Vt.; excitation wavelength, 420 to 470 nm; emission wavelength, 490 to 530 nm). To enumerate the total number of bacteria, samples were stained with DAPI (4′,6-diamidino-2-phenylindole) and viewed with a DAPI filter set (7). Staining with DAPI interfered with enumeration by GFP, and the two enumerations could not be done with the same slides. Fixation of samples with Formalin did not affect GFP detection, on the basis of a comparison of fixed and unfixed controls.

The five treatments were as follows: controls with no added bacteria, autoclaved stream water with unmodified JM109 (parent), whole stream water with the parent, autoclaved stream water with plasmid-bearing JM109 (GEM), and whole stream water with the GEM.

Total numbers of bacteria from DAPI counts revealed that the parent and GEM exhibited similar mortality rates (Fig. 1A). The total number of bacteria in both the parental and GEM treatments declined in whole stream water but was more stable in autoclaved water. The uniformity of response of the parent and GEM indicates that the presence of pGFP did not adversely affect the fitness of the host strain.

The number of green fluorescent cells in the autoclaved and whole stream water followed a temporal pattern similar to that of the total bacterial number (Fig. 1B). No green fluorescent cells were detected in the parental or control samples.

There was a disparity between GFP and DAPI counts in the autoclaved water containing the GEM (Fig. 1). The counts from the two methods at time zero were the same, while at other times DAPI counts exceeded GFP counts. There are three possible explanations for this observation. First, some

* Corresponding author. Phone: (330) 672-3788. Fax: (330) 672-3713. Electronic mail address: LLEFF@KENTVM.KENT.EDU.

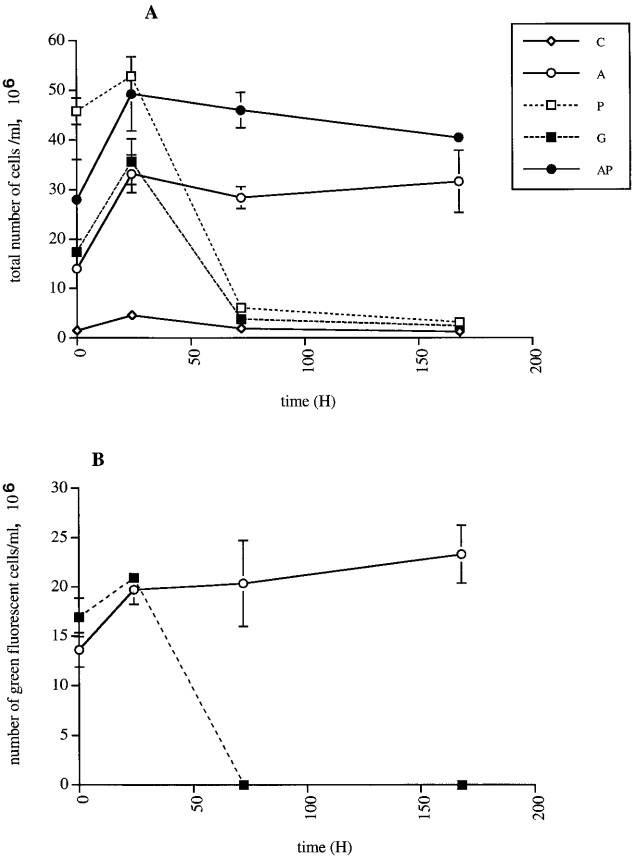

FIG. 1. Number of cells, as determined by microscopy, after staining with DAPI (A) or viewed without staining with the GFP filter set (B). Treatments: C, control; A, autoclaved water with GEMs; P, whole water with parent; G, whole water with GEMs; AP, autoclaved water with parent. Values are means (n = 3), with standard errors indicated by error bars.

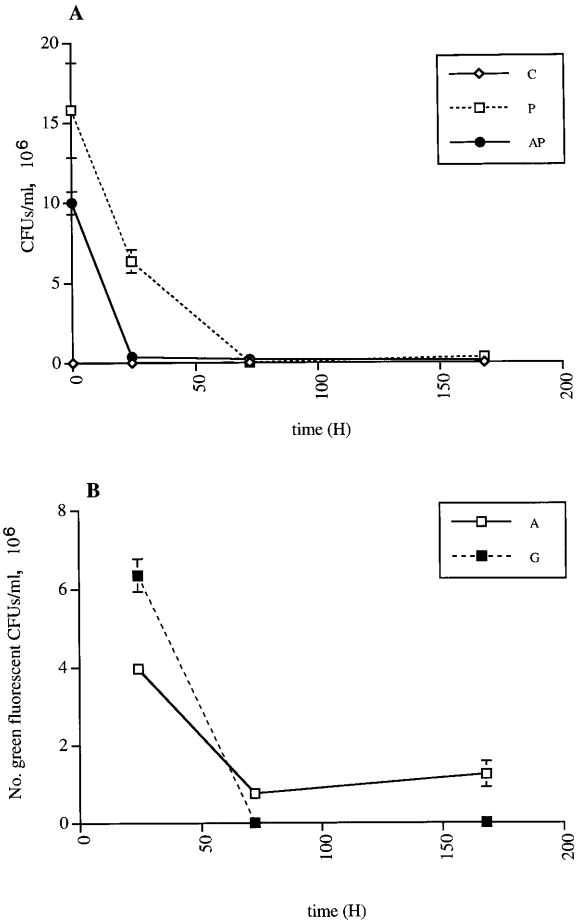

FIG. 2. (A) Number of CFU after plating on Luria agar; (B) number of green fluorescent CFU on Luria agar with ampicillin. Treatments: C, control; A, autoclaved water with GEMs; P, whole water with parent; G, whole water with GEMs; AP, autoclaved water with parent. Values are means (n = 3), with standard errors indicated by error bars.

cells that originally contained pGFP may have lost their plasmid or stopped making GFP under the low-nutrient conditions of the stream water. Second, DAPI may stain some cells which are no longer viable and have lost their DNA (11) and GFP. Third, there may be a time lag between production of GFP and fluorescence.

The number of CFU in flasks containing the parent strain declined sharply in both the autoclaved and whole water (Fig. 2A). Declines of the parent in autoclaved water based on CFU were much greater than declines based on DAPI counts (Fig. 1A), indicating that the parental strain rapidly lost culturability. Alternatively, the disparity between DAPI and CFU counts may indicate the presence of undegraded, nonviable cells (ghosts [11]).

The number of green CFU in the GEM-treated flasks declined over time in both autoclaved and whole stream water (Fig. 2B). The abundance of green CFU was substantially lower than the abundance of green cells visible microscopically, indicating a substantial loss of culturability like that of the parental strain.

E. coli did not survive in whole stream water in the presence of predators and competitors. Its persistence in autoclaved stream water suggests that its survival in nature is limited by biological interactions. One factor leading to its success is that autoclaving enhances the lability of dissolved organic carbon, perhaps providing a better carbon source for *E. coli* than streams do.

Other studies examining survival of *E. coli* in stream water support the findings of this project. The number of *E. coli* HB101 CFU declined after 3 days in whole stream water but was stable in filtered water (2). The authors concluded that biological factors led to the decline in *E. coli* abundance. Similarly, McFeters and Terzieva (6) found that CFU of *E. coli* E7 declined in stream water. In both of these previous studies, *E. coli* was enumerated by plating.

Use of the GFP gene allowed rapid and precise identification of GEMs in water by plating and microscopy. GEMs maintained pGFP, on the basis of the observation that all CFU of GEMs from autoclaved water were green fluorescent. Most individual GEMs still contained GFP at the end of the incubation, as determined microscopically. Further research is needed to determine if they continued to produce GFP during the experiment or if initial GFP stocks in the cells persisted for the duration of the experiment. Use of GFP in this experiment revealed that *E. coli* may persist in a nonculturable state after it is no longer detectable as CFU.

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