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Fate of pBR322 DNA in a wastewater matrix

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Recombinant organisms used in biopharmaceutical production processes are destroyed prior to environmental release into a private or municipal wastewater treatment system. However, concern over the fate of recombinant DNA used in these processes may adversely affect product regulatory approval. This study examined the fate of DNA from the plasmid pBR322 in an activated sludge-derived matrix. DNA suitable for PCR amplification was extracted from the activated sludge matrix and a 1042-bp fragment from pBR322 rapidly decreased in concentration from 0 to 2 h after it was spiked into the activated sludge matrix at an initial DNA concentration of 25 ng ml^{-1}. While some evidence of the 1042-bp fragment was observed at 4 h, no evidence of amplified DNA was observed at 6 h. Plasmid DNA in buffer that served as a positive control exhibited no significant reduction in concentration over time. The intensity of each DNA band over the first 4 h was analyzed. A linear regression of the natural log transformation of these results yielded a mean first-order rate constant of 3.55 h^{-1} and half-life of 0.2 h. This study demonstrated that recombinant DNA released from industrial processes into wastewater treatment systems should be rapidly degraded.

Keywords: sludge; wastewater; DNA; pBR322; genetically manipulated organisms

Introduction

Genetically manipulated organisms (GMOs) that contain recombinant DNA are used in many commercial processes to produce biological control agents, chemicals, pharmaceuticals, and agricultural chemicals. Processes that use such GMOs have engineering and operational control measures that minimize the chance of accidental discharge to the environment. However, to provide an estimate of the ecological risk from such an accidental release, an understanding of the environmental fate of both the GMO and its DNA should be conducted.

While regulatory goals for such testing vary greatly depending upon the specific use of the GMO, those processes that produce pharmaceuticals are regulated by the US Food and Drug Administration (FDA). The FDA requires that an environmental assessment be conducted for each pharmaceutical product marketed in the United States as described in the Code of Federal Regulations, Title 21 Part 25 [4]. As part of that environmental assessment, an understanding of the fate and effects of both the GMO and its product may be required [5].

While engineering controls significantly limit any release of viable GMOs, it is possible that recombinant DNA from the process or the organisms themselves could survive heat or chemical treatment of the process effluent. In the vast majority of cases, these process effluents are subsequently discharged to either private or municipal wastewater treatment plants which could provide opportunity for genetic exchange of recombinant DNA.

Based upon the lack of peer-reviewed studies of recombinant DNA fate in activated sludge matrices and the desire to develop a PCR-based assay that could quickly examine process effluent recombinant DNA in wastewater, the current investigation was initiated using pBR322 DNA as a model.

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Materials and methods

DNA

Plasmid DNA pBR322 [3] was obtained from Gibco BRL (Grand Island, NY, USA).

Inoculum source and pre-treatment

Activated sludge was collected from the aeration basin of the Valley Forge Municipal Utilities Authority Wastewater Treatment Plant, Valley Forge, PA, USA. The study was replicated three times using different batches of activated sludge from the same plant with the total suspended solids concentration averaging 2.5 g L^{-1}. Sludge was aerated upon arrival at the laboratory and was used within 24 h after collection. For each experiment, approximately 800 ml of fresh sludge was placed in a Waring laboratory blender and blended at high speed for approximately 30 s to dissociate bacteria from the sludge flocs. After blending, sludge was allowed to stand for approximately 15 min to settle sludge flocs. The supernatant fluid was then decanted and immediately used.

Test system and DNA spiking

Test samples consisted of 80 ml activated sludge supernatant fluid in 100-ml glass milk dilution bottles. DNA was introduced into test vessels by first diluting pBR322 DNA into 20 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 8) and then adding this mixture into the 80 ml of pretreated sludge. This spiking procedure was done so that the pBR322 would be homogenously distributed before possible sorption to organic matter in the test mixture. Positive control samples contained 100 ml of TE buffer and 10 μl

pBR322. The final concentration of pBR322 in the positive control and test samples was 25 ng ml^{-1}. Negative control samples consisted of a 100-ml aliquot of the collected sludge supernatant fluid that was pipetted into a glass milk dilution bottle.

Sampling

Sampling of the vessels began immediately after spiking and at time intervals up to 52 h. One-milliliter samples were removed from the negative control, positive control, and test samples and immediately placed into microcentrifuge tubes. Microcentrifuge tubes were then placed immediately in a 85°C water bath for 30 min to minimize nuclease activity. Following this treatment, microcentrifuge tubes were placed in a −80°C freezer until DNA isolation.

DNA isolation

Samples for DNA isolation were first thawed at room temperature and then 500 μl was removed for DNA extraction using the Elu-Quik DNA Isolation Kit (Schleicher & Schuell, Keene, NH, USA). DNA was precipitated with ethanol prior to resuspension in TE.

PCR conditions and primers

PCR amplification was conducted using primers PBR1 and PBR2. The DNA sequence for PBR1 was 5′-GCGACCGAGTTGCTCTTGCCCGGCG-3′ and for PBR2 the sequence was 5′-CAAGGAGATGGCGCCCAACAGTCC-3′. These primers were used to amplify a 1042-bp fragment of pBR322 located between the 3890 and 573 base pairs [2]. This fragment contains part of the ampicillin and tetracycline resistance genes. PCR was performed using a DNA thermal cycler (Perkin Elmer System 2400), and the GeneAmp reagent kit (Perkin-Elmer, Norwalk, CT, USA). Samples were subjected to 95°C for 60 s, followed by 30 cycles each of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final cycle at 72°C for 7 min. Magnesium chloride was added to a final concentration of 2.5 mM.

DNA quantification and degradation kinetics

DNA was separated using electrophoresis in a 1% agarose gel at 70 volts and visualized by staining with ethidium bromide. Gel image photographs were digitized using a Bio-Rad (Hercules, CA, USA) Model GS-700 Imaging Densitometer and Molecular Analyst software.

Results

DNA suitable for PCR amplification was extracted from the activated sludge matrix during all experiments. The 1042-bp fragment from pBR322 was observed to decrease rapidly in concentration from 0 to 2 h after spiking it into the activated sludge matrix (Figure 1). A faint band was also observed at 4 h that was not visible in the image of the gel presented in Figure 1. No visible evidence of amplified DNA was observed at 6 h. The pBR322 DNA incubated in TE buffer exhibited no significant reduction in concentration over time (Figure 1). No amplified DNA was observed in negative controls (data not shown).

The intensity of each PCR DNA band over the first 4 h of each experiment was analyzed and the image-adjusted volume calculated using Bio-Rad Molecular Analyst

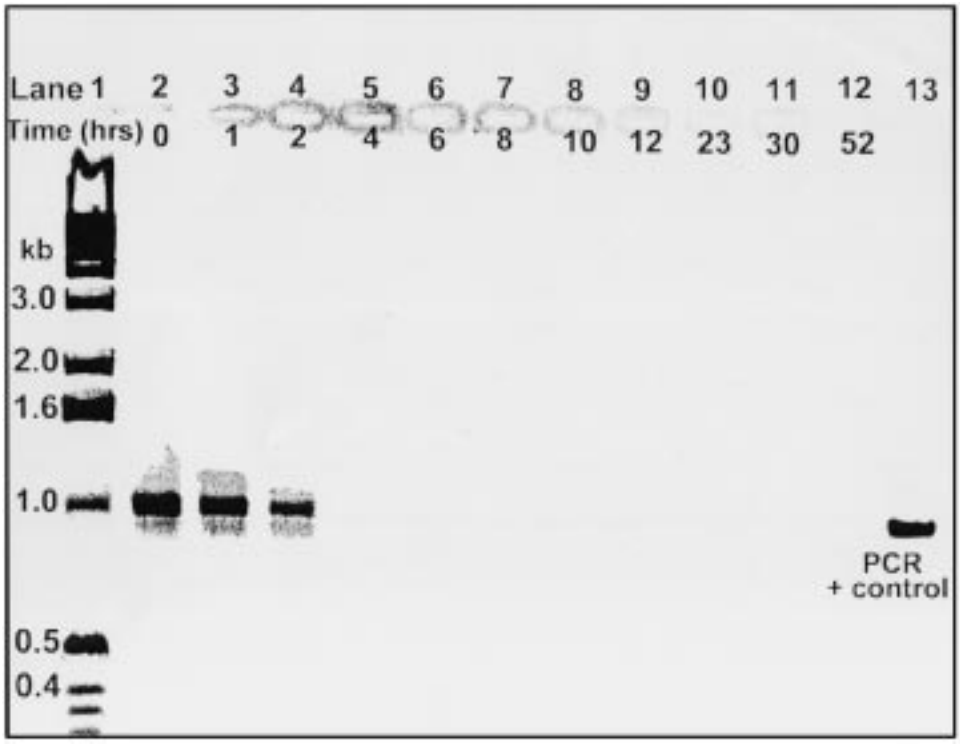

Figure 1 Enhanced contrast image of agarose gel containing pBR322 DNA PCR products extracted at various time intervals from a wastewater-derived matrix. Lane 1: molecular weight markers: lanes 2–12: extracted PCR-amplified 1.04-kb pBR322 DNA PCR products after varied exposures to wastewater as shown below lane numbers; lane 13: amplified positive control, amplified fragment from pBR322 in TE buffer.

software. A mean first-order rate constant of 3.55 h^{-1} (SD = 0.82, $n = 3$) and a half-life of 0.2 h were calculated from the linear regression of the natural log transformation of these results using pseudo first-order kinetics.

Discussion

GMOs are commonly used to produce substances of commercial interest and their use will most certainly increase with time. While engineering controls will significantly limit any release of viable GMOs, it is plausible that recombinant DNA from the process or the organisms themselves could survive heat or chemical treatment of the process effluent. In the vast majority of cases, these process effluents are subsequently discharged wastewater treatment plants that could provide opportunity for genetic exchange of recombinant DNA. Few published studies have examined the actual fate of recombinant DNA in wastewater matrices.

While some investigations have demonstrated the importance of examining the fate of intact bacterial strains, few studies have examined the wastewater fate of extracellular DNA used in an industrial process. Phillips *et al* examined the degradation of pBR322 in a Michigan trickling filter plant over a period of 30 min [6]. Approximately 5 μg of unmodified pBR322 was added to 100 μl wastewater and sampled over time. Samples were then examined via agarose electrophoresis and the time required for the plasmid to be converted from covalently closed circular (CCC, supercoiled) to open circular (OC) or linear DNA was quantified. The half life for this transition was approximately 5 min in wastewater and it was concluded that a CCC DNA molecule is unlikely to survive wastewater treatment because of both endonucleolytic and exonucleolytic attack. However, the recombinant DNA concentration used [6] was unrealistically high.

The current investigation determined that pBR322 DNA at an initial concentration of 25 ng ml^{-1} was rapidly degraded in an activated sludge matrix with a mean half-life of approximately 12 min. DNA was not detectable by PCR 6 h after inoculation. This rapid degradation may be attributed to both endo- and exonuclease activity in the wastewater-derived matrix. This result is in general agreement with Phillips *et al* [6] although the use of PCR in the present study allowed DNA detection at a far lower concentration increasing the amount of time the DNA could be detected.

Recently, Alvarez et al [1] studied the fate of plasmid pWTAla5′ DNA in *E. coli* DH1 cells in distilled, tap, marine, and river water, using dot blot hybridization as well as bacterial conjugation. DNA concentration remained relatively constant for up to 7 days in distilled water whereas DNA in tap water exhibited a sharp decrease in concentration after 7 days. DNA in marine water was degraded after 12 h while DNA in river water was degraded after 18 h. Samples subjected to PCR analysis showed no amplification indicating complete DNA degradation. The data obtained during this investigation support Alvarez *et al*'s findings of complete DNA degradation in other matrices.

In conclusion, although other investigations have examined plasmid degradation in other sterile and non-sterile wastewater matrices, this study utilized DNA amplification of activated sludge inoculated samples at nominal DNA concentrations far less than those of other reported studies and determined that DNA concentration rapidly decreased to below the limit of PCR detection.

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