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Potential for gene transfer from recombinant *Escherichia coli* K-12 used in bovine somatotropin production to indigenous bacteria in river water

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SUMMARY

This study examined the transfer of the plasmid pBGH1, an expression vector for bovine somatotropin (BST), from *Escherichia coli* K-12 strain W3110G [pBGH1] to indigenous microorganisms present in flasks containing Missouri River water. Strain LBB269 is a nalidixic acid-resistant derivative of W3110G which was used as a plasmid-free control strain in these studies. Water samples were inoculated with strains W3110G [pBGH1] and LBB269; after 21 days of incubation the number of viable colony-forming units (CFU) of W3110G [pBGH1] and LBB269 were reduced from an initial level of about 1×10^7 CFU per ml to less than 1 CFU per 100 ml. At this time indigenous microbes resistant to both ampicillin and tetracycline (the antibiotic resistance markers on pBGH1) were isolated from 100 ml of water from each of the flasks inoculated with either strain W3110G [pBGH1] or LBB269. Plasmid DNA was isolated from these organisms and examined for sequences containing the gene for BST from pBGH1, using a polymerase chain reaction (PCR) assay. As expected, the day 0 sample from the flask inoculated with *E. coli* K-12 strain W3110G [pBGH1] gave a positive PCR response and the day 0 sample from the flask inoculated with *E. coli* K-12 strain LBB269 gave a negative PCR response. All of the day 21 samples containing indigenous microbes isolated from flasks that were inoculated with either W3110G [pBGH1] or LBB269 were negative in the PCR assay, indicating that the target sequence from pBGH1 was not present in any of these indigenous microorganisms. The results of this particular assay indicate that pBGH1 or the portion of pBGH1 including the BST structural gene had not been transferred from W3110G [pBGH1] to indigenous microbial inhabitants of the Missouri River water flasks during this study.

INTRODUCTION

Escherichia coli K-12 strains have been used extensively as hosts for recombinant plasmids, both for research as well as commercial purposes. Commercial production of proteins and other products from recombinant strains of *E. coli* usually involves fermentation of a strain containing an expression plasmid in volumes from hundreds of liters to tens of thousands of liters, with culture densities attaining 1×10^9 to 1×10^{10} cells per ml [5,6]. The prospect of up to 1×10^{17} (or more) recombinant *E. coli* cells being inadvertently released into the environment during some type of catastrophic industrial accident has ignited considerable interest in the consequences of such an event. This interest includes asking whether recombinant *E. coli* K-12 strains can survive in natural environments and whether the recombinant plasmid they contain can be transferred to indigenous inhabitants of these environments. The natural environments usually studied are water, soil, sewage, and the mammalian intestinal tract.

The fate of *E. coli* K-12 strains in the environment has been examined by a variety of investigators [3], although none of these studies employed strains actually used for commercial production purposes. *E. coli* K-12 strains remained viable for approx.15 days in water, 20 days in soil, 10 days in sewage, and 3–6 days in the intestinal tract of normal hosts. The transfer of recombinant plasmids from strains of *E. coli* K-12 to indigenous inhabitants of water, soil, sewage, or normal intestinal tracts, in their natural environments, has never been demonstrated.

E. coli K-12 strain W3110G [4] containing the pBR322-based plasmid pBGH1 [7] is used by Monsanto Company for the large-scale production of bovine somatotropin (BST) [5,6]. Although a number of systems have been designed to prevent the escape of this recombinant microorganism from fermentation areas, it was of interest

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to determine the fate of W3110G [pBGH1] in environments outside the production plant. In a previous paper we described studies on the survival of this strain in water [2]. This report describes a study of the potential for pBGH1 to be transferred from the *E. coli* strain W3110G [pBGH1] to indigenous microorganisms present in Missouri River water. The objective of this study was to determine whether the plasmid pBGH1, or the portion of pBGH1 including the BST structural gene, was transferred from the *E. coli* K-12 strain W3110G [pBGH1] to indigenous microorganisms in a flask of Missouri River water. Initially, W3110G [pBGH1] was present at levels about 100-fold greater than the indigenous microorganisms in order to increase the likelihood of such a transfer event. The flasks were incubated for 21 days at 26 ± 2 °C. As the final step in the study, all of the indigenous microorganisms in 100 ml of Missouri River water were examined for the target sequences indicative of gene transfer. In order to maximize the possibility of detecting what was expected to be at most an extremely rare transfer event, a two-step screen with a very low detection limit, based on a polymerase chain reaction (PCR) assay, was developed.

MATERIALS AND METHODS

Media and reagents

Levine Eosin Methylene Blue (EMB) Agar and Plate Count Agar media were obtained from Difco Laboratories (Detroit, MI). For this study, the Plate Count Agar was referred to as tryptone-yeast extract-glucose (TYG) agar. The plates contained the indicated antibiotics tetracycline, ampicillin, nalidixic acid, and cycloheximide (Sigma Chemical Co., St. Louis, MO) at final concentrations of 10, 100, 200, and 250 mg, respectively, per liter. TYG plates containing tetracyline, ampicillin, and cycloheximide were labeled TYG TAC. TYG plates containing cycloheximide were labeled TYG C. EMB plates containing tetracycline, ampicillin, and cycloheximide were labeled EMB TAC; EMB plates containing nalidixic acid and cycloheximide were labeled EMB NC; and EMB plates containing nalidixic acid, ampicillin, and cycloheximide were labeled EMB NAC.

Collection of water

The Missouri River water was collected at river mile 54, a point about 12 miles upstream of the Monsanto Life Sciences Research Center in Chesterfield, MO. The collection site was within the Weldon Spring Wildlife Area, public land owned and operated by the State of Missouri. This site was chosen both because of ease of public access and because this section of the Missouri River is undeveloped. A detailed chemical and microbiological description of this water has been published [2].

Growth of E. coli *K-12 strains*

E. coli K-12 strains LBB269, LBB269 [pBGH1], and W3110G [pBGH1] were grown in 15-liter Biolafitte fermenters [4]. When the cultures were in the mid-exponential phase of growth (at an optical density at 550 nm of approx. 40, corresponding to about 2×10^{10} CFU per ml), 50-ml samples were collected.

Inoculation and incubation of water samples

Ten-ml aliquots from the three fermentation samples were added to 90 ml of Missouri River water, and then 10 ml of these diluted samples were added to 990 ml of Missouri River water. This effected a 1000-fold dilution of the fermentation samples into the river water and resulted in inoculum densities of about 10^7 CFU per ml. For each strain, three flasks of river water were inoculated. The flasks were placed on a rotary shaker at 200 rpm in a 26 °C incubator.

Preparation of day 0 PCR samples

A 1-ml sample was removed from each of the three flasks that had been inoculated with W3110G [pBGH1]. These samples were serially diluted and plated in triplicate on TYG TAC plates. The resulting colonies on each plate of the 1×10^4 dilution were suspended in 2 ml of sterile deionized water. The cells from the three plates for each flask were pooled in a sterile tube, and then frozen at −20 °C until used. This yielded three day 0 samples, one from each flask that had been inoculated with W3110G [pBGH1]. Each sample was composed of the cells from about 3000 colonies.

Also, a 1-ml sample was removed from each of the three flasks that had been inoculated with LBB269; from these samples 0.2- and 0.1-ml aliquots were plated in triplicate. The resulting colonies on all nine plates of the 5×10^0 and 1×10^{-1} dilutions, respectively, were suspended in a total of 6 ml each (2 ml per three plates per dilution) and frozen at −20 °C until used. This yielded two day 0 samples, each one a mixture of cells from either the 5×10^0 or the 1×10^{-1} dilution from all three flasks that had been inoculated with LBB269; these samples were composed of the cells from about 900 or 450 colonies, respectively.

Plating of LBB269 [pBGH1] on day 15

About 110 ml of water from each of the three flasks that had been inoculated with LBB269 [pBGH1] were removed and filtered through Whatman No. 1 filter paper. Five 20-ml aliquots of this prefiltered water were filtered through sterile 0.45-μm filters, and the filters were placed on EMB NAC plates.

Plating of day 21 samples

Samples from all nine flasks that had been inoculated with *E. coli* were prefiltered through Whatman No. 1 filter papers to remove suspended matter. Four 25-ml aliquots of each of these nine prefiltered samples were filtered through sterile 0.45-μm filters. The 12 filters obtained from the samples of the flasks that had been inoculated with W3110G [pBGH1] were placed on EMB TAC plates, the 12 filters obtained from the samples of the flasks that had been inoculated with LBB269 were placed on EMB NC plates, and the 12 filters obtained from the samples of the flasks that had been inoculated with LBB269 [pBGH1] were placed on EMB NAC plates.

Preparation of day 21 cell suspensions for PCR analysis

On day 21, four 25-ml aliquots of each of the six prefiltered samples (described above) of flasks that had been inoculated with W3110G [pBGH1] and LBB269 were filtered through sterile 0.45-μm filters, and the 24 resulting filters placed on TYG TAC plates. The resulting colonies of each of these 6 sets of four filters were suspended in a total of 10 ml of deionized water.

Preparation of cell suspension for PCR spiking experiment

About 2200 ml of water were pooled from the three uninoculated flasks of Missouri River water and prefiltered through sterile Whatman No. 1 filter paper to remove sediment. This prefiltered water was then filtered through 20 sterile 0.45-μm filters, 100 ml of water per filter, and the 20 filters placed on TYG TAC plates. The resulting colonies, about 100 per filter, were suspended in a total volume of about 25 ml of sterile deionized water.

Incubation of agar plates

TYG plates were incubated at $26 \pm 2\,^{\circ}C$ for approx. 48 h, and EMB plates were incubated at $37 \pm 2\,^{\circ}C$ for approx. 24 h.

Isolation of DNA from the cell samples

DNA was isolated from the cell samples by the procedure of Birnboim and Doly [1].

PCR analysis of the DNA samples

The PCR assays were done using a Perkin-Elmer/Cetus DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT). The DNA samples isolated from spiked and unspiked samples were in a final volume of 100 μl. From each of these DNA samples, 10 μl were used in each PCR assay. The reaction mixture contained 24 μl of each dNTP (dATP, dCTP, dGTP, and dTTP, from 10 nmol per μl stocks), 744 μl of deionized water, 120 μl of 10 × reaction buffer, 60 μl each of primers PBR1 and PBR2 (from 4.4 pmol per μl stocks), and 6 μl of AmpliTaq® DNA polymerase (from 5 units per μl stocks). The dNTP stocks, 10 × reaction buffer, and AmpliTaq® DNA polymerase were purchased from Perkin-Elmer Corp. The primers PBR1 and PBR2 were purchased from the Midland Certified Reagent Company (Midland, TX). To 90.5-μl aliquots of the reaction mixture described above were added 10 μl of each DNA sample. These mixtures were placed in the DNA Thermal Cycler, programmed for 30 cycles of 1 min at $94\,^{\circ}C$, 1 min at $60\,^{\circ}C$, and 1 min at $72\,^{\circ}C$.

The PCR primers used for this assay were named PBR1 and PBR2. Primer PBR1 has the sequence 5′–GCG-ACCGAGTTGCTCTTGCCCGGCG–3′, and primer PBR2 has the sequence 5′–GCAAGGAGATGGCGC-CCAACAGTCC–3′. Primer PBR1 hybridizes to pBGH1 at coordinates 5020–5044, oriented clockwise, and primer PBR2 hybridizes to pBGH1 at coordinates 1676–1700, oriented counter-clockwise (Fig. 1). The PCR fragment produced from a pBGH1 template using these two primers is 2178 base pairs in length and includes the entire BST structural gene and flanking elements, including small portions of the structural genes encoding resistance to ampicillin and tetracycline.

Polyacrylamide gel electrophoresis of DNA samples following PCR amplification

Eight μl of running dye (0.25% bromophenol blue and 1% EDTA in 50% glycerol, pH 8.0) was added to a 40-μl aliquot of each amplified DNA sample. These samples and a DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) were loaded into the wells of a 6.8% polyacrylamide gel. A constant current of 40 mAmp was applied to the gel until the bromophenol blue dye front neared the bottom. The gel was then placed in an ethidium bromide staining solution (25 mg ethidium bromide in a liter of buffer containing 10.8 g Tris base, 0.93 g disodium EDTA, and 5.5 g boric acid) for at least 5 min, and then destained with distilled water for at least 15 min. The gel was placed onto the surface of a ultraviolet transilluminator and photographed.

RESULTS AND DISCUSSION

Viable counts on day 0

A viable count of uninoculated Missouri River water on day 0 revealed that aerobic microorganisms were present at a level of about 2.2×10^5 CFU per ml. Cycloheximide was included in all media in order to inhibit the growth of fungi, and to reduce the swarming tendency of certain microorganisms. The flasks of Missouri River water which had been inoculated with the *E. coli* K-12 strains contained approx. 2×10^7 CFU per ml, nearly 100-fold higher than the concentration of indigenous microor-

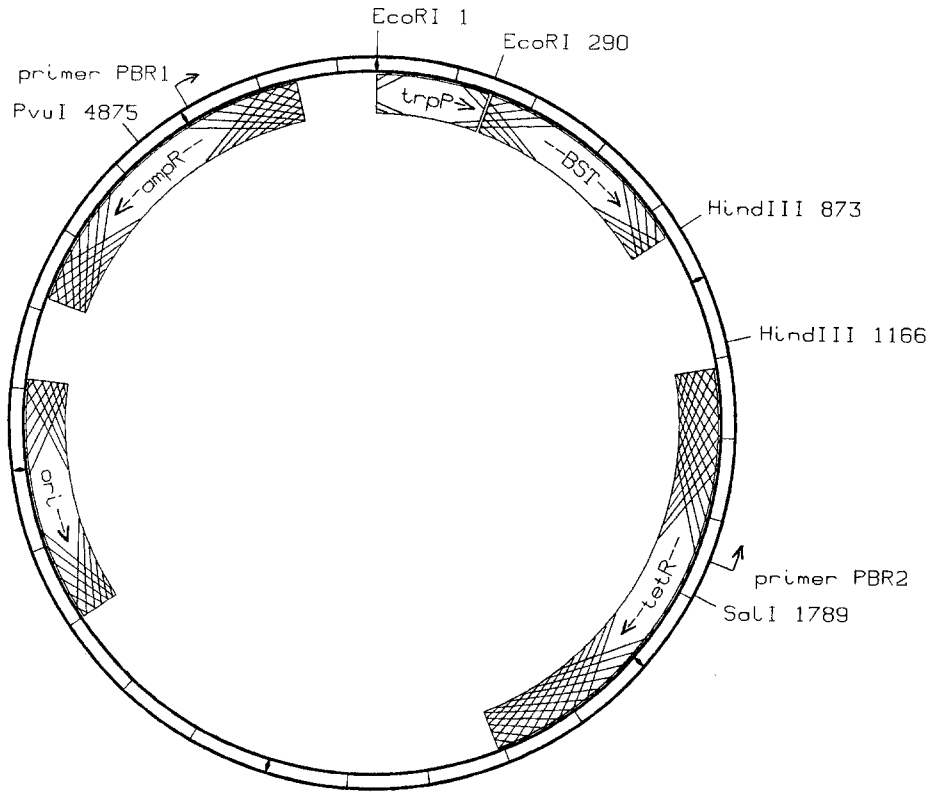

Fig. 1. Map of the plasmid pBGH1, showing hybridization sites of the primers PBR1 and PBR2. Included are the recognition sites for several key restriction endonucleases, as well as the locations of the tryptophan promoter (trpP), the bovine somatotropin structural gene (BST), the origin of plasmid DNA replication (ori), and the structural genes encoding resistance to ampicillin (ampR) and tetracycline (tetR). Arrows indicate the orientation of each of these plasmid elements.

ganisms. Thus, the day 0 plates from these flasks were comprised almost totally of colonies of *E. coli*.

Viable counts on day 15

In order to avoid false positive reactions in the PCR assay, it was necessary to ascertain when the number of viable CFU of inoculated *E. coli* had dropped to less than 1 in 100 ml of water. For all three strains, the levels had dropped to about 100 CFU per ml in 6 days (data not shown), a result consistent with our previous studies with this system [2]. Viable counts of *E. coli* strain LBB269 [pBGH1] were determined from much larger volumes of water on day 15. The flasks containing *E. coli* strain LBB269 [pBGH1] were checked because these flasks were not going to be used in the gene transfer experiment. No *E. coli* colonies were found in 100-ml samples of these flasks, suggesting that the level of LBB269 [pBGH1] had dropped below 1 CFU per 100 ml. This represents a 10^9-fold decrease in the viability of this *E. coli* strain over a period of 15 days in this flask.

Viable counts on day 21

Before initiating the gene transfer experiment, viable counts of aerobic microorganisms were determined on day 21 from all 12 of the flasks. The three flasks of uninoculated Missouri River water contained aerobic microorganisms at an average level of about 1.0×10^5 CFU per ml. The average level of aerobic microorganisms was 2.7×10^5 CFU per ml for the three flasks that had been inoculated with W3110G [pBGH1], 6.4×10^5 CFU per ml for the three flasks that had been inoculated with LBB269, and 1.9×10^5 CFU per ml for the three flasks that had been inoculated with LBB269 [pBGH1]. The essentially identical number of CFU in the inoculated and uninoculated flasks suggested that the added *E. coli* K-12 cells were no longer viable.

As a further check for viable *E. coli* cells present in these flasks, 100-ml samples were checked from each of the nine flasks that had been inoculated with *E. coli* cells; EMB media containing the appropriate antibiotics were used. About 800 colonies were obtained from each 100-ml

sample. These colonies were examined to see whether any *E. coli* colonies were present. On EMB media, these *E. coli* strains form characteristic small, round, raised Lac^+ colonies with a distinct metallic green sheen. No such colonies were seen with any of the samples, confirming that the number of *E. coli* in these flasks of Missouri River water had fallen to less than 1 CFU per 100 ml.

Among the colonies on the EMB TAC plates (plated from the flasks that had been inoculated with W3110G [pBGH1]) there were 14 round, flat black Lac^+ colonies without any hint of a metallic green sheen. While these colonies did not appear coliform in any way, it was decided to examine them as well isolated colonies in case the crowding on the filter was affecting their morphology. All 14 of these colonies were picked with a sterile loop and streaked upon the appropriate media. In each case, in the least dense areas of the streaks there were several well isolated colonies. None of these were *E. coli* colonies.

From all of these results, we concluded that by day 21 of this study the number of *E. coli* in these flasks of Missouri River water had fallen to less than 1 CFU per 100 ml, and that the gene transfer study could be conducted on these samples without any *E. coli* cells causing a false positive PCR assay.

PCR analysis

Six day 21 cell suspensions were prepared, three from the flasks that had been inoculated with W3110G [pBGH1] and three from the flasks that had been inoculated with LBB269. To verify that none of these cell suspensions contained *E. coli* cells, a series of test plates were performed on EMB TAC or EMB NC, respectively. Since the four filters from which each of these six samples had been obtained (see Materials and Methods) had originally contained a total of about 800–1000 colonies, any contaminating *E. coli* colonies would have been present at a level of at least 0.1%, easily detectable under these plating conditions. No *E. coli* colonies were seen on any of the test plates.

These six cell suspensions were diluted to give six individual cell pellets of approximately the same size as that normally used in the small scale DNA isolation procedure employed in this study. DNA was isolated from both the five frozen day 0 cell pellets and the six day 21 cell pellets and subjected to PCR analysis (Fig. 2). Each DNA sample was assayed three times. All three independent PCR analyses gave the same results (data not shown). That is, the day 0 DNA samples from the flasks inoculated with W3110G [pBGH1] were positive for the presence of pBGH1, while the day 0 DNA samples from the flasks inoculated with LBB269 were negative (Fig. 2).

The three day 21 samples obtained from the negative control flasks that had been inoculated with LBB269 gave, as expected, a negative PCR assay. The three day 21 experimental samples obtained from the flasks that had been inoculated with W3110G [pBGH1] also gave a negative result in the PCR assay (Fig. 2). These results indicate that pBGH1 had not been transferred from W3110G [pBGH1] to indigenous microorganisms in the Missouri River water.

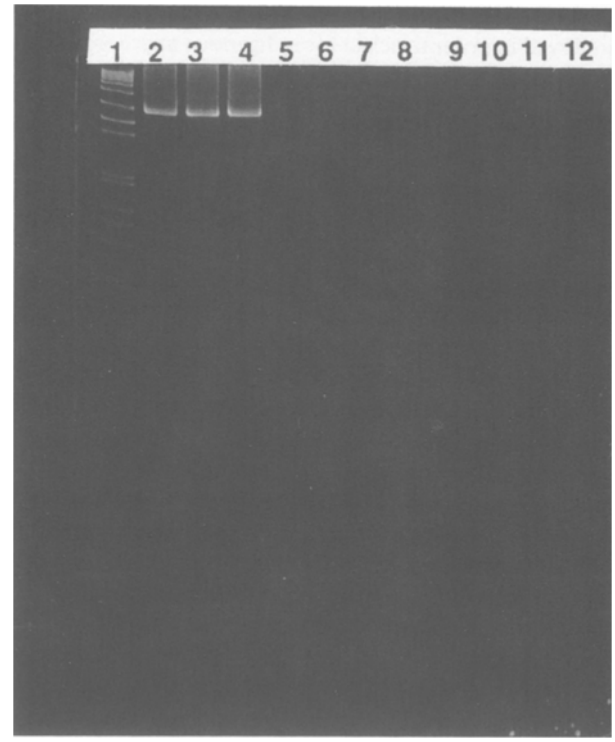

Fig. 2. Polyacrylamide gel electrophoresis of samples from the experimental PCR assays. The PCR fragment produced from a positive sample should be 2178 base pairs in length. Lane 1 contains a DNA size standard; the fragment seen in lanes 2, 3 and 4 is running next to the 2036 base pair fragment in this DNA size standard, as expected for a positive PCR assay. Lanes 2, 3 and 4 contain aliquots of the PCR reactions run on the three DNA samples isolated from cells taken on day 0 from each of the three flasks that had been inoculated with W3110G [pBGH1]. Lanes 5 and 6 contain aliquots of the PCR reaction run on the two DNA samples isolated from cells taken on day 0 from the flasks that had been inoculated with LBB269. Lanes 7, 8, and 9 contain aliquots of the PCR reactions run on the three DNA samples isolated from cells taken on day 21 from each of the three flasks that had been inoculated with W3110G [pBGH1]. Lanes 10, 11, and 12 contain aliquots of the PCR reactions run on the three DNA samples isolated from cells taken on day 21 from each of the three flasks that had been inoculated with LBB269.

Detection limit of the PCR assay

The detection limit of the PCR assay was determined by performing a 'spiking' experiment. Colonies were isolated from the uninoculated flasks of Missouri River water;

TYG plates containing ampicillin and tetracycline were employed in order to select for indigenous microorganisms that might contain plasmids and to mimic as closely as possible the conditions of the gene transfer study itself. It was felt that the maximum interference with the PCR assay would be obtained from indigenous organisms containing plasmid DNA. A cell suspension was prepared from these colonies. W3110G [pBGH1] was used to provide the pBGH1-bearing cells in the spiking experiment.

An overnight culture of W3110G [pBGH1] was serially diluted in sterile Luria broth, and the *E. coli* cells and the indigenous bacterial cells from the suspension were mixed to produce ten spiked cell suspensions, corresponding to 1.0 ml, 0.5 ml, and from 1×10^{-1} to 1×10^{-8} ml of *E. coli* per 2 ml of indigenous bacterial cells. These spiked cell suspensions were placed in sterile 15-ml tubes, the volumes adjusted to 10 ml with sterile Luria broth, and the DNA isolated and subjected to the PCR assay procedure. The PCR assay was positive with DNA samples obtained from the first eight spiked tubes (i.e., down to 1×10^{-6} ml *E. coli*) and negative in spiked tubes 9 and 10 (Fig. 3).

Viable counts of the culture of W3110G [pBGH1] that had been used for the spiking and the suspended colonies of indigenous microorganisms from the filters revealed that the culture of W3110G [pBGH1] contained about 6×10^8 CFU per ml, and the suspension of indigenous microorganisms contained about 4.6×10^9 CFU per ml. From these results it was possible calculate that in the last PCR-positive spiked tube there was 1 *E. coli* CFU per 1.5×10^7 indigenous microorganisms CFU. Thus, the PCR method employed in this study was capable of detecting as few as 1 W3110G [pBGH1] CFU per 1.5×10^7 CFU of ampicillin and tetracycline resistant indigenous microorganisms from the Missouri River. Presumably, this means that this PCR assay could detect one positive colony among up to 1.5×10^7 colonies.

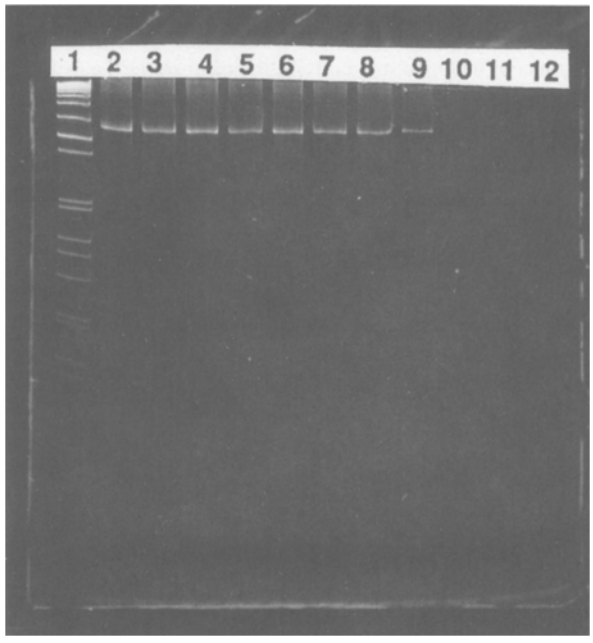

Fig. 3. Polyacrylamide gel electrophoresis of samples from the PCR spiking experiment. Lanes 2–11 contain aliquots of the PCR reactions run on the ten DNA samples isolated from the ten spiked tubes described in the text. That is, lane 2 corresponds to spike tube No. 1, lane 3 corresponds to spike tube No. 2, and so forth through lane 11, which corresponds to spike tube No. 10. Lane 12 contains a PCR control (no added DNA).

Transfer of pBGH1 was not detected

On day 21 of the study the three flasks of Missouri River water that had been inoculated with W3110G [pBGH1] contained an average of 2.7×10^5 CFU of indigenous microorganisms per ml. For each sample in this study all of the organisms in 100 ml of water, 2.7×10^7 CFU, were examined by a two-step screening process involving first isolating microorganisms resistant to ampicillin and tetracycline and then isolating DNA from those microorganisms and subjecting it to a PCR assay.

The four filters used to generate each of the samples in the experimental PCR assays only contained a total of 800–1000 colonies. This is easily within the limit of detection for this assay, as determined above. The negative result of the gene transfer study performed on these samples indicates that the plasmid pBGH1 was not transferred from the *E. coli* K-12 strain W3110G [pBGH1] to indigenous microorganisms in a flask of Missouri River water.

It was very encouraging to find that using a two-step gene transfer assay, which could detect one positive recipient cell among 27 million cells, we were unable to demonstrate gene transfer from the recombinant *E. coli* to indigenous microbes. The absence of gene transfer in this study demonstrates that such events, if they even occur at all, must occur at a frequency of less than 1 in 2.7×10^7 in these flasks.

The fact that gene transfer was not observed in this system is probably a reflection of several factors. Transfer of pBR322-based plasmids, such as pBGH1, requires the presence of conjugational and mobilization plasmids, high cell densities and metabolic activities of donor and recipient, and optimum temperatures (27–37 °C) [3]. Such conditions are seldom if ever encountered in the environment. Thus it is not surprising that the transfer of recombinant plasmids from strains of *E. coli* K-12 to indigenous inhabitants of water, soil, sewage, or normal intestinal tracts, in their natural environments, has never been demonstrated.

PCR is proving to be a very useful tool in tracking small

numbers of specific organisms in complex environments. For example, Steffan and Atlas [8] were able to detect a single cell of *Pseudomonas cepacia* in 1 g of river sediment containing about 1×10^6 CFU of indigenous bacteria. Tsai and Olson [9] were able to detect *E. coli* at a level of about 3 cells per g of soil containing about 3×10^4 CFU of indigenous bacteria. Both of these studies employed PCR primers directed against regions which occurred at multiple locations in the genomes of the target organisms; there were 15–20 copies of *P. cepacia* target and seven copies of the *E. coli* target. Similarly, the PCR primers used in the present study were directed against a multiple target by virtue of the fact that it was on a high copy number plasmid. The lower level of detection achieved in this study is presumably due to the less complex environment (water vs. soil or sediment) allowing concentration of microorganisms by filtration.

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